

# Techniques for the Investigation of Fish Physiology

(Rukovodstvo po metod ke issledovaniya fiziologii ryb)

*A Collection of Articles*

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## Contents

PREFACE	1
P A Korzhuev METHODS OF STUDY OF BLOOD IN FISH	2
N V Puchkov THE WHITE BLOOD CELLS	11
I A Balakhnin BLOOD GROUPS OF FISH AND METHODS OF THEIR DETERMINATION	18
N V Puchkov IMMUNOBIOLOGICAL REACTIONS	20
V A Remorov ELECTROCARDIOGRAM RECORDING IN LONG TERM EXPERIMENTS	25
N S Stroganov METHODS OF STUDY OF RESPIRATION IN FISH	27
V S Ivlev DETERMINATION OF ACTIVE METABOLISM	80
G S Karzinkin and M N Krivobok BALANCE SHEET EXPERIMENTS ON NITROGEN METABOLISM OF FISH	91
N S Stroganov METHODS FOR AMMONIA DETERMINATION USED IN STUDIES ON FISH METABOLISM	106
M N Krivobok and O I Tarkovskaya DETERMINATION OF FAT IN THE FISH BODY	112
D A Shubnikov METHODS FOR FAT DETERMINATION UNDER FIELD CONDITIONS	119
G S Karzinkin and O I Tarkovskaya DETERMINATION OF CALORIC VALUE OF SMALL SAMPLES	122
B V Krayukhin METHODS OF INSTALLING CHRONIC FISTULAS IN THE DIGESTIVE TRACT OF FISH	125
V A Pegel THE USE OF METT S ROOS FOR THE SIMULTANEOUS DETERMINATION OF ENZYME COMPOSITION ENZYME ACTION AND MOTOR ACTIVITY OF THE DIGESTIVE TRACT IN FISH IN CHRONIC EXPERIMENTS	138

V.A. Pegel'. METHODS OF STUDY OF THE DIGESTIVE PROPERTIES OF PANCREATIC JUICE AND BILE IN FISH IN LONG-TERM EXPERIMENTS . . . . .	141
V.A. Pegel'. STUDIES ON ENZYME COMPOSITION, ENZYME ACTIVITY AND MOTOR ACTIVITY IN VARIOUS SECTIONS OF THE DIGESTIVE TRACT OF FISH IN LONG-TERM EXPERIMENTS . . . . .	143
E.A. Veselov. METHODS OF OSMOTIC REGULATION STUDIES IN FISH . . . . .	146
A.G. Ginetinskii, V.F. Vasil'eva, M.G. Zaks, Yu.V. Natochin and M.M. Sokolova. METHODS OF STUDY OF OSMOREGULATING SYSTEMS IN FISH . . . . .	168
T.I. Privol'nev. DETERMINATION OF FREEZING POINT OF BLOOD SERUM BY MEANS OF A MICROELECTROTHERMOMETER . . . . .	178
N.V. Puchkov. METHODS OF STIMULATION OF OVULATION IN FISH . . . . .	183
L.C. Voronin and Yu.A. Kholodov. EXPERIMENTAL METHODS OF STUDY OF FISH BEHAVIOR . . . . .	184
N.V. Prazdnikova. METHODS OF STUDY OF CONDITIONED REFLEXES IN FISH . . . . .	200
V.R. Protasov. STUDIES ON VISION IN FISH . . . . .	217
C.A. Malyukina. METHODS OF STUDY OF HEARING IN FISH . . . . .	230
M.P. Aronov. METHODS OF STUDY OF TASTE AND SMELL IN FISH . . . . .	236
C.A. Malyukina. METHODS OF STUDY OF THE LATERAL LINE ORGAN OF FISH . . . . .	252
V.I. Gusel'nikov. METHODS OF STUDY OF BIOELECTRIC BRAIN ACTIVITY . . . . .	258
G.A. Malyukina. OPERATIONS ON BRAIN OF FISH . . . . .	267
B.P. Mantel'sel' and V.R. Protasov. EFFECT OF LIGHT INTENSITY ON THE BEHAVIOR OF FISH . . . . .	271
V.S. Ivlev. METHODS OF DETERMINATION OF OPTIMAL LIGHT CONDITIONS . . . . .	278
S.G. Zusser and O.A. Sokolov. METHODS OF STUDY OF THE REACTION OF SHOAL FISH TO LIGHT . . . . .	285
E.A. Veselov. SIMPLEST METHODS FOR STUDIES ON SOME REFLEXES OF FISH LARVAE AND FRY . . . . .	297
LIST OF ABBREVIATIONS . . . . .	313

## PREFACE

The First All-Union Symposium on Fish Physiology held in 1956 acknowledged that "For the successful accomplishment of the tasks facing the fishing industry, further and more detailed studies on fish physiology are required. Fish physiologists must undertake studies directly concerned with the fishing industry" (resolution of the Symposium). Progress in this field had been hampered by the absence of a manual of methods for studying fish physiology. The Symposium urged the need for this deficiency to be made good by the compilation and publication of such a manual. An Editorial Board for this purpose was set up which included E.N. Pavlovskii (Member of the Academy of Sciences), V.A. Pegel', P.A. Korzhuev, T.I. Privol'nev, N.V. Puchkov, B.V. Krayukhin, G.S. Karzinkun, L.G. Voronin, N.L. Gerbil'skii, E.M. Malikovs, and G.A. Malyukina.

The Ichthyological Board was asked to take the necessary steps to secure the publication of this manual.

The Editorial Board chosen by the Symposium has now accomplished this task. The present treatise comprises 33 chapters on methods used in the investigation of fish physiology including the study of: blood, respiration, metabolism, digestion, osmotic regulation, and, finally, methods for the experimental study of the higher nervous activity of fish. In addition to methods suitable for well-equipped physiological laboratories, methods for field studies are also given. These methods should be of use not only to physiologists, but also to ecologists, and to less highly-trained personnel employed in the fishing industry.

The chapters are arranged so that the necessary data is easily accessible to specialists in any particular field without their needing to consult additional literature.

Methods for the study of fish metabolism by the use of radioactive or stable isotopes or special biochemical analytical methods have not been included as these are adequately covered already in the specialized literature.

## METHODS OF STUDY OF BLOOD IN FISH

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Detailed comparative studies on the blood of man and of agricultural animals have given grounds for assuming that blood is a unique "mirror" in which all vital processes taking place in the organism are reflected. This is the basis of a hypothesis which is at present under study by zoologists, according to which blood properties are directly correlated with breeding properties and fertility of animals. Blood tests are among the main standards for the assessment of breeding properties and fertility in animals.

Fish blood has not been studied fully, in spite of the fact that a detailed knowledge of blood properties is indispensable for the understanding of biological processes in the different fish species. Very accurate methods are now available for determining the total amount of blood in the organism, and of its plasma components and formed elements. Essential in this respect are the methods for determining the number of red blood cells (erythrocytes), hemoglobin, and total blood volume, since these serve as indexes of the level of energy-yielding processes taking place in fish and hence of the activity of the fish species in question.

### ERYTHROCYTE COUNT METHODS

The method of direct counting of erythrocytes per given volume of blood (mostly per cubic millimeter) is used (Predtechenskiy, 1960). Since erythrocyte concentration in the blood is very high, blood samples should be diluted 100 or 200 times prior to counting. The blood drop so diluted is placed in a counting chamber and the erythrocytes are counted in several squares of the ruled surface. Blood volume corresponding to each square is known. Having counted the number of erythrocytes in a given number of squares, one can calculate the number of erythrocytes per cubic millimeter of blood. Determination of the number of erythrocytes should be carried out in the following order.

1. Obtaining of blood. Blood is taken by means of a Pasteur pipet the thin end of which is introduced into blood vessels located in the region of the gill cover or into the caudal artery. The skin is punctured by the sharp end of the capillary tube. Having obtained the required amount of blood, the latter is placed on a watchglass and used for filling the diluting pipets.
2. Diluting pipet (Fig. 1, A) - for blood dilution. This is a pipet with an egg-shaped bulb located nearer to one of the pipet's ends. The long, graduated stem of the pipet serves for drawing blood by suction. It

usually has two marks 0.5 and 1.0. The pipet has the figure 101 engraved above the bulb, denoting that the entire pipet holds 100 times more liquid than the stem to the mark 1.0 (or 200 times more than the stem to the mark 0.5). The egg-shaped bulb contains a small glass ball to facilitate mixing the blood mixture. The tip of the long stem is pointed, the opposite end is fitted to hold a rubber tube for suction. In good diluting pipets, the lumen of the stem is not too wide. Both the upper mark on the stem and that above the bulb should be as close to the bulb as possible because the fluid between each of these marks and the bulb cannot be mixed and the greater the volume of this liquid, the greater the error of determination.

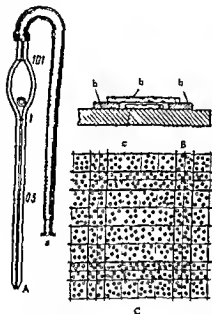


FIGURE 1 Diluting pipet for counting chamber (for counting blood cells)

A - diluting pipet B - counting chamber C - counting chamber filled with blood under microscope a - rubber tube, b - glass platforms c - coverglass

3 Diluting fluid The most widely used diluting fluids are Hayem's fluid or sodium chloride solution. Isotonic solution of sodium chloride is usually used, but it is better to employ a hypertonic solution, to prevent hemolysis. Hayem's fluid consists of mercuric chloride 5g, sodium chloride 10g, and sodium sulfate 37.5g in 1000ml water.

4 Counting chambers The counting chamber (Fig. 1, B, C) consists of a heavy glass slide, in the center of which a glass platform with an engraved ruling is etched. Along both sides of the middle platform ("floor piece") with the ruling there are two other platforms rising exactly 0.1 mm above the central one. These platforms are covered by a thick coverglass ground perfectly plane, so that when the coverglass is in place the height of the chamber is exactly 0.1 mm. For this purpose a special thick glass with ground edges is used. This coverglass should adhere tightly to the platforms, as judged by radial bands, the so-called Newton rings. The best counting chamber is that of the Burkert type. The results obtained with this counting chamber are more accurate than those obtained with other types of counting chambers (e.g. Zeiss counting chamber).

5 Rulings. Counting chambers with different rulings are known (ruling of Thomas-Zeiss, Turk, Burkert, Neubauer, Predtechenski and Goryaev). Rulings of all types consist of a square surface subdivided into numerous small squares (varying numbers, depending on the type of counting chamber) grouped differently in the different counting chambers (Fig. 2). The small squares in all rulings are of identical dimensions: 1/20 mm wide, and 0.1 mm high (when the coverglass is in place). The volume corresponding to the area of one small square is equal to 1/4000 mm<sup>3</sup>. The large square contains 16 small squares. The different rulings vary in the number of large squares.

and in their location. Counting chambers of **Burker** and **Turk** have 144 large squares, **Predtechenski's** ruling has 100 large squares, and the **Thoma-Zeiss** ruling - 16 large squares.

6. Filling the diluting pipet and charging the counting chamber. The pipet with blood is filled to the 1.0 or 0.5 mark. The blood adhering to the tip is wiped off with cotton wool and plunged into the diluting fluid and the fluid is drawn. At this stage it is best to hold the pipet nearly vertically in order to avoid inclusion of a large air bubble in the bulb (occasionally an air bubble may ascend together with the fluid and by-pass the bulb). The fluid is drawn up to the 101 mark (the upper mark above the bulb). Then the rubber tube is removed, both ends of the pipet are closed with the thumb and third finger, and the pipet is vigorously shaken in all directions for 3 - 5 min. Some laboratories are equipped with mechanical shakers which ensure uniform mixing. This of course gives better results, since thorough mixing of blood in the diluting pipet is a prerequisite for accurate blood cell counts.

After the blood has been mixed thoroughly by shaking, it is immediately placed into the counting chamber with the coverglass in place. To be sure that the blood in the counting chamber comes from the bulb of the diluting pipet and not from the stem, the first several blood drops from the pipet are discarded. Thereafter, the diluting pipet is shaken several times and the counting chamber charged by letting the blood drop from the pipet flow along the edges of the coverglass. The fluid will run under the coverglass by capillary action, and fill the chamber space. The corpuscles will settle in a short time and counting may be commenced. After counting, the diluting pipets should be rinsed with distilled water and dried with the aid of a rubber bulb, the counting chamber should also be rinsed with distilled water and dried with cotton wool or gauze.

7. Counting erythrocytes. Erythrocytes are usually counted in the small squares. In order to avoid counting one and the same erythrocyte twice, the following rule is adopted: all cells within the square and on the borderlines are counted. The latter are counted only when their greater part is within the square; erythrocytes which touch the upper and right sides should be counted as though they were within the square (not less than  $\frac{1}{4}$  of the erythrocyte should lie within the square). Cells with their greater part outside the square are not counted.

Erythrocytes are usually counted in eighty small squares (5 large squares). In **Burker's** ruling, small squares are arranged between large squares. There are 12 large and 13 small squares in a single row. Erythrocytes are counted in all small squares in 6 alternate rows, and in two squares of the seventh row.

The number of erythrocytes per  $1\text{ mm}^3$  of blood is determined in the following way: The volume of the small square =  $1/20 \times 1/20 \times 1/10 = 1/4000\text{ mm}^3$ . Thus, the number of cells per  $1\text{ mm}^3$  of blood for rulings of all types can be calculated from the following formula:

$$x = \frac{\text{the number of cells counted} \times 4000 \times \text{blood dilution}}{\text{the number of small squares counted}}$$



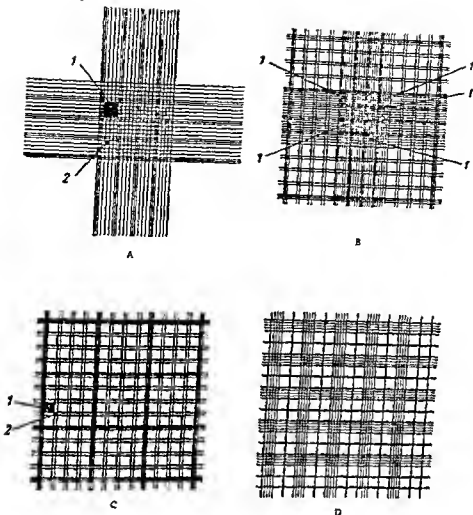


FIGURE 2 Rulings of counting chambers

A - Thomas's counting chamber; B - Turk's counting chamber; C - Barker's counting chamber, D - Goryaev's counting chamber 1 - large square, 2 - small square

Dividing the number of cells by the number of small squares, one obtains the number of cells per one small square, i.e. per  $1/4000 \text{ mm}^3$ . Hence there are 4000 times more cells per  $1 \text{ mm}^3$ . Thus the number of cells found in one small square is multiplied by 4000. In addition, blood dilution must be corrected. For example, 100 erythrocytes were counted in 80 small squares. Blood was diluted 1:200. Thus the total erythrocyte count per one cubic millimeter of blood amounted to-

$$\frac{100 \times 4000 \times 200}{80} = \frac{8,000,000}{80} = 1,000,000 \text{ erythrocytes}$$

Usually when blood is diluted 1 : 200, and 80 small squares are counted, one may omit the constant figures  $\frac{4000 \times 200}{80} = 10,000$  and simply multiply the number of erythrocytes counted in 80 small squares by 10,000.

The accuracy of the method for counting erythrocytes depends on a number of factors (the accuracy of taking the blood sample, accuracy of diluting the blood sample, thoroughness of mixing). The error in making a blood count (in the case of human erythrocytes - which have been counted most frequently) may range from 3.0 to 10%; the error in counting leukocytes may range from 7.0 to 30%.

#### DETERMINATION OF VOLUME OF BLOOD CORPUSCLES

The relative volume of blood corpuscles ("corpuscular volume percentage") as determined by the hematocrit is a very important criterion. In addition, it may serve as a verification of the number of erythrocytes counted with the aid of counting chambers.

Hematocrit tube (Fig. 3) is made of heavy glass with a narrow (2-3 mm) inner diameter. There are 100 graduations on the tube wall. The hematocrit tube is filled with blood from a Pasteur pipet, and centrifuged for 45 min at 3000 rpm. The level of packed erythrocytes is read and centrifugation is continued for an additional 15 min. Centrifugation is discontinued when the second reading coincides with the first one.

There are various types of hematocrit tubes, which differ from each other in form and size. The best hematocrit tube is that proposed by Wintrobe (1933).

Under normal conditions, in freshly captured fish the corpuscular volume percentage as determined by the hematocrit ranges from 20 to 35%, i. e. there are 20-35 volumes of cells for each 100 volumes of blood. Exceptionally this figure rises to 50% (pelamid blood).

If blood clots are present in the hematocrit tube, they lead to serious errors (high hematocrit values). It is recommended to perform hematocrit determinations in duplicates, and to centrifuge the samples to constant volume.

The use of the hematocrit may also indicate the presence or absence of hemolysis in the blood sample. Hemolysis cannot be ascertained by counting erythrocytes.

#### DETERMINATION OF HEMOGLOBIN CONTENT

Several tests are available for the determination of hemoglobin content. The most widespread and simplest, but at the same time the least accurate, is the colorimetric method of Sahli (1909).

The Sahli method. This method is based on the conversion of blood hemoglobin into acid hematin with hydrochloric acid, and matching the color of this acid hematin solution with that of a standard solution.

The Sahli hemometer is a frame with three compartments for test tubes. Two test tubes are fixed and contain acid hematin



FIGURE 3  
Hematocrit  
tube

solution of known concentration. The third test tube (between the two fixed test tubes with standard solution) is graduated and provided with two scales. One has 140 graduations, and the other scale (on the opposite side of the test tube) gives a reading of hemoglobin content in grams per 100 cc of whole blood, 100 per cent on this scale corresponds to 16.0 or 17.3 g of hemoglobin (depending on the model). Hemometer readings may be expressed in percentages and in grams per 100 cc of whole blood. If the values are expressed in percentages, it should be stated to how many grams 100% correspond.

Hemometers are also provided with a graduated pipet with 20 cubic millimeter mark.

To determine the hemoglobin content a drop of blood is obtained and drawn into the pipet to the 20 mm<sup>3</sup> mark. Decinormal solution of hydrochloric acid is poured into the graduated tube to the mark 10. The blood from the pipet is blown into the hydrochloric acid solution in the graduated tube and the pipet rinsed several times with the hydrochloric acid solution. After mixing with hydrochloric acid, the blood assumes a brown color similar to that of the standard solutions. The fluid of the graduated tube is diluted with distilled water, drop by drop, until it has exactly the same color as the standard solutions. The graduation corresponding to the surface of the fluid indicates the concentration of hemoglobin. Hemoglobin content of fish ranges from 30 to 90%, and 5 to 14 g per 100 cc of whole blood.

The accuracy of hemoglobin determination is affected by the following factors:

1. The time elapsed from the moment of mixing blood with hydrochloric acid to the reading. It should be mentioned that the color intensity of hematin solution is not stable, thus, in order to obtain comparable results all determinations should be made after a strictly determined time, between 3-5 min. When the Zeiss hemometer is used, the reading should be made exactly after 5 min (exactly 5 min after mixing with hydrochloric acid, the color of hematin matches that of the standard solutions).

2. Since fish erythrocytes contain nuclei, the turbidity formed by the nuclei which are insoluble in hydrochloric acid must be accounted for. The greater the number of erythrocytes in the blood, the more marked the turbidity. Analysis of blood of a male sturgeon showed that the hemoglobin concentration amounted to 12.7 g per 100 cc of whole blood. After centrifugation, however, this value fell to 9.8 g per 100 cc of whole blood or by 22.9%. The hemoglobin concentration of sevruga fell from 15.8 to 12.5 g per 100 cc of whole blood, i.e. by 18.0%. The hemoglobin content of sheathfish was determined as 8.4 g per 100 cc of whole blood. Following centrifugation, this value fell to 5.4 g per 100 cc of whole blood, i.e. by 17.4%. On the average (data from numerous determinations), centrifugation brings about a 18-20% decrease in the value of hemoglobin content, this figure is too large to be ignored.

More accurate methods are those based on the determination of the oxygen capacity of blood, or of blood iron content (iron is an integral part of the hemoglobin molecule). These methods, however, are more laborious, more complex and not always suitable in field conditions.

There is a definite correlation between the volume of erythrocytes and the blood hemoglobin content. Owing to this, one may determine the volume, hemoglobin content and hemoglobin concentration of one erythrocyte. These data are important since they provide an indirect confirmation of the

accuracy of our determinations. Thus, for example, it was found that 1 mm<sup>3</sup> of sheathfish blood contains 1,400,000 erythrocytes; hematocrit value, 23.0%; hemoglobin content - 54 g per 100 cc of whole blood.

The volume of one erythrocyte can be determined from the following formula:

$$\frac{\text{hematocrit reading}}{\text{number of erythrocytes}} \times 10 = \text{Volume } (\mu^3)$$

$$\frac{23.0}{1.4} \times 10 = 164.3 \mu^3.$$

The hemoglobin content of a single erythrocyte is

$$\frac{5.4}{1.4} \times 10 = 39 \text{ micromicrograms}$$

Hemoglobin concentration per one erythrocyte amounts to

$$\frac{5.4}{23.0} \times 100 = 23.5\%$$

Since it is thought that the nucleus of fish erythrocytes, like that of other vertebrates, is devoid of hemoglobin, the true hemoglobin concentration is somewhat higher when the volume occupied by the nucleus is corrected for. According to Wintrobe, the nucleus of the fish erythrocyte occupies about 16.0% of the total volume of the erythrocyte.

#### DETERMINATION OF FISH BLOOD VOLUME

Determination of fish erythrocyte properties with the aid of the above formulas, like that of erythrocytes of other animals, is widely applied by various investigators, and in our opinion is of great importance in the general blood tests of the different fish species. Although it has many advantages, the above method is inadequate for comprehensive studies on fish erythrocytes. This method would be satisfactory were the blood volume of fish constant, like that of other animals. Literature data, however, indicate that blood volume of fish fluctuates widely, depending on the age of the fish, the season and the general condition of the animal. Hematological studies in various fish species cannot be considered as complete unless the total blood volume is determined. This, in combination with the data obtained from analysis of one blood drop (hemoglobin, erythrocyte count, mean corpuscular volume) enables the investigator to obtain more accurate estimates that are obtained from the analysis of one blood drop only.

1. The direct method for determining blood volume. The animal is bled to exhaustion, its organs ground and the remaining blood extracted from the ground tissues. This method is suitable for work with small animals. From small fish the blood sample is usually taken only for the determination of hemoglobin or hematocrit, intestinal and gallbladder contents are removed, all organs are ground, mixed with distilled water to remove the remaining blood, and kept for one day in the cold (at + 2°C, + 4°C). Then the tissues are filtered off and the volume of the filtrate measured, its hemoglobin content or the volume of erythrocytes in it is determined.

The filtrate should be transparent. If the hemoglobin content or the volume of erythrocytes in the whole blood and in the extract from the ground organs and tissues is known, the total blood volume can be calculated.

2. **Perfusion method.** This is based on removal of blood by perfusion. This method is used in studies of large fish. First, a precisely known volume of blood is obtained and analyzed for hemoglobin concentration. Perfusion is then carried out. The volume of perfusate is accurately determined, and its hemoglobin concentration ascertained. The total blood volume is calculated from the dilution of the perfusate in relation to the whole blood.

Perfusion is carried out as follows: 0.8% solution of sodium chloride is placed in a transfusion bottle provided with a rubber tube and glass cannula to be inserted into the aorta.

The experimental fish is weighed and tied to a frame consisting of two wooden planks joined to each other at an obtuse angle. The fish is fixed with its belly up. The skin and muscles are incised in the heart region, between the anterior fins and gill covers. The portion of the vessel between gills and aortic bulb is exposed. The exposed portion is tied with a ligature, and the peripheral and central ends are compressed with two clamps. An incision is then made, and the glass cannula is inserted into the vessel (directed towards the heart). After the cannula has been fixed inside the vessel, the clamp is removed. The outflowing blood is collected in a test tube. In this blood, the hemoglobin content or the erythrocyte volume is determined. When the blood flow has ceased, the cannula is taken out and inserted into the peripheral end of the aorta. Then the cannula is connected with the rubber tube of the perfusion bottle filled with physiological sodium chloride solution, and the clamp on the peripheral end of the aorta is removed. Perfusion of the vascular system commences; the perfusate flows through the cut in aortic bulb. Perfusion is continued until the perfusate becomes colorless. This is usually achieved after 30 minutes or one hour of perfusion. Perfusion is then discontinued. The fish is weighed, and the volume of perfusate, its hemoglobin concentration or the erythrocyte volume in the perfusate are determined.

For the determination of hemoglobin concentration in the perfusate the color of the perfusate is compared with that of suitably diluted blood. For this purpose a series of test tubes with various blood dilutions is prepared. To a series of colorless glass test tubes containing 2 ml of distilled water each, the following amounts of whole blood (in ml) are added with the aid of a micropipet: 0.09; 0.08; 0.07; 0.06; 0.05; 0.04; 0.03; 0.025; 0.020; 0.015; 0.010; 0.007; 0.006, and 0.005 ml. This corresponds to blood dilutions from 1:22 to 1:400. 2 ml of the perfusate are added to a similar test tube and its color is compared with that of the standard solution. In this way the dilution of blood in the perfusate is determined. Since the volume of the perfusate is known, the amount of blood present in the perfusate can be easily calculated.

Example: a sazan weighing 980 g was studied. Ten ml of blood were obtained from the aorta. The perfusate volume was 310 ml. The color intensity of the perfusate corresponded to that of the test tube containing 0.07 ml blood in 2 ml of water. If 2 ml contains 0.07 ml of blood, 310 ml of perfusate will contain

$$\frac{0.07 \times 310}{2} = 10.85 \text{ ml of blood.}$$

Hence the total blood volume of the sazan is  $10.85 + 10 = 20.85$  ml or 21.68 g which comprises 2.4% of the total body weight.

Let us examine another example in which the blood volume of a sheath-fish was determined from hematocrit values.

Experiment, February 2, 1949. Sheathfish (*Silurus glanis*), weight 650 g, body length 47 cm. Whole blood obtained from aorta, 9.0 ml. Volume of perfusate, 250 ml. Volume of erythrocytes in whole blood (hematocrit) 17.0%. Volume of erythrocytes in perfusate (hematocrit) 0.9%. The dilution of blood in the perfusate is thus  $17.0 : 0.9 = 18.8$  - fold dilution. This means that 250 ml of perfusate contains  $250.0 : 18.8 = 13.29$  ml of whole blood. Hence the total blood volume of the sheathfish was  $13.29$  ml +  $9.0$  ml =  $23.3$  ml. Assuming that the specific gravity of blood is 1.04, the total weight of blood is  $22.3 \times 1.04 = 23.2$  g or 3.6% of the total weight of the sheathfish.

In relatively large fish the volume of blood may also be determined with the aid of vital stains introduced into the blood stream (heart). The total blood volume is calculated from the extent of dilution of the dye in the blood stream as compared to the concentration of dye in the initial solution (Prosser and Weinstein, 1950).

Determination of blood volume in fish, although important for its own sake, does not accurately reflect the characteristic ecological properties of the fish examined, because their blood may have varying hemoglobin concentrations. If the hemoglobin content of the whole blood and the total blood volume of the fish in question are known, the total hemoglobin content of the blood and the amount (in grams) of hemoglobin per kg body weight of the fish can be calculated. The latter index is of special importance, since it enables one to compare the total hemoglobin content in various fish species. Thus, if the total blood volume of a sheathfish weighing 650 g is 22.3 ml and the hemoglobin concentration is 5.45 g per 100 ml of blood, then there are 1.87 g of hemoglobin per kg body weight in the fish in question.

The ecological properties of various fish species can be judged by the concentration of hemoglobin in their organisms.

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## THE WHITE BLOOD CELLS

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### BLOOD LEUKOCYTE COUNT

The normal number of leukocytes in fish blood exceeds considerably that in the blood of mammals and in man, amounting, in some species of bony fish, to more than 100,000 leukocytes per  $\text{mm}^3$  of blood. The number of leukocytes in one and the same fish species varies greatly depending on the age, season and maturity of the sex glands.

Blood for counting of leukocytes may be taken from the heart after exposing the thoracic cavity, or from the branchiostegal vessels. The best method is the one in which blood is obtained from the caudal artery. For this purpose, the fish is wrapped in a towel so that the tail and anal fin will remain free, the fish is then placed with its belly upwards. Even more convenient is to wedge the fish between two parallel thin boards fixed on a steady base (Fig. 1).

The slippery posterior anal fin is wiped with a dry swab and the skin in this region is punctured with a needle. In the hole produced the end of a Pasteur pipet is inserted and plunged deeper and deeper into the muscles by slowly rotating the pipet. This is done until the pipet's tip reaches the vertebral bones. The rotating movement of the pipet tears the caudal artery present on the ventral side of the vertebral column, and blood begins to flow into the pipet. Having secured an adequate amount of blood, the pipet is taken out and the blood blown onto a watchglass. Owing to the rapid clotting of fish blood, the entire procedure of obtaining blood should be carried out as rapidly as possible. From the watchglass the blood is drawn into a diluting pipet used for counting mammalian erythrocytes and diluted 100 times (see the preceding chapter of P. A. Korzhuev). Blood is drawn up to the 1.0 mark and then diluted with special diluting fluids for vital staining of leukocytes. For this purpose, the following solutions A and B are prepared, prior to counting.

#### Solution A

Neutral red . . . . .	25 mg
Sodium chloride . . . . .	0.6 g
Distilled water . . . . .	100 ml

#### Solution B

Crystal violet . . . . .	12.0 mg
Sodium citrate . . . . .	3.5 "
Formalin . . . . .	0.4 ml
Distilled water . . . . .	100 "

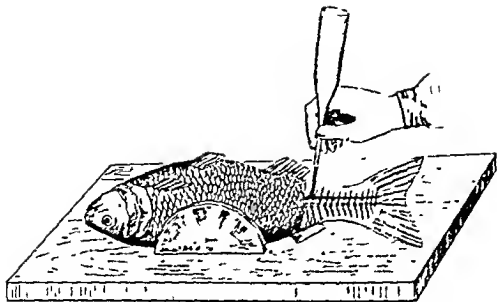


FIGURE 1. Obtaining of blood from fish

For better solubility of the dye, the salt present in each of the solutions should be added after the dye has completely dissolved.

The solutions should not be stored for long periods of time. Solution A may be stored for one day. Solution B may be stored for about one week.

Before the studies, solutions A and B are poured into shallow porcelain crucibles. Blood is drawn into the diluting pipet up to the 1.0 mark, this being followed by drawing solution A approximately to one-half of the volume of the bulb of the pipet. The tip of the diluting pipet is then plunged into the crucible with solution B and drawn up to the 101 mark (above the bulb). Having mixed the blood with the dye, the diluting pipet is left for 5-10 min in a horizontal position and again mixed. The first 2-3 drops are discarded, and the following drop is placed in a leukocyte counting chamber.

The rulings of the counting chambers consist of small and large squares. The area of large squares in which leukocytes are counted is  $1:25 \text{ mm}^2$ . The groupings of large and small squares differ in the different types of counting chambers (see the preceding paper by P.A. Korzhuev).

Hold the diluting pipet in an inclined position and touch its tip to the angle between the edge of the coverglass and one of the projecting ends of the central platform with ruling. The diluted blood will run under the coverglass by capillary action, and fill up the space between the lower surface of the coverglass and the surface of the floor piece with the ruling. Microscopic examination reveals that the fluid is distributed over squares with the total area of  $1:25 \text{ mm}^2$ . Since the height of the fluid column is 0.1 mm, the fluid volume will be  $1:250 \text{ mm}^3$ .

The nuclei of leukocytes are vitally stained a dark violet-red, and the protoplasm remains pink. In the erythrocytes only the nuclei are stained slightly, thus distinguishing them from the leukocytes.



Leukocytes are counted in 8 - 10 large squares of the ruling of the counting chamber. The average number of leukocytes in one square is multiplied by 250 and by the dilution factor (i.e. by 100). In this way the number of leukocytes per cubic millimeter of blood is calculated.

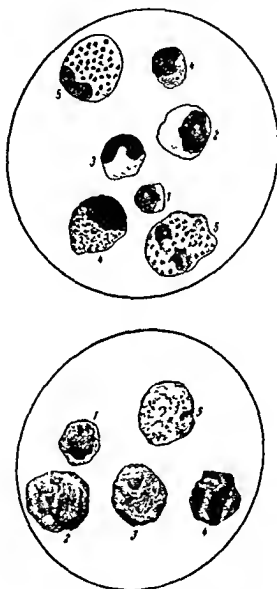


FIGURE 2. Different types of leukocytes in fish

Top - blood of stellate sturgeon bottom - blood of carp  
1 - lymphocytes 2 - monocytes 3 - polymorphonuclear  
leukocytes; 4 - neutrophils 5 - eosinophils

The number of red blood cells may be determined at the same time in the same chamber. For this purpose, erythrocytes are counted in 80 small squares and the average number of erythrocytes per small square is multiplied by 400 and then by the dilution factor (i. e. by 100).

#### DIFFERENTIAL BLOOD LEUKOCYTE COUNT

White blood cells of fish consist of several kinds of cells differing from one another in form and affinity for dyes (Fig. 2). Bony fish show five main types of leukocytes: three types without granular cytoplasm, and two with acidophil or neutrophil granularity of the protoplasm. The agranulocytic types of fish leukocytes include lymphocytes, monocytes and polymorphonuclear leukocytes. Lymphocytes are small cells whose cytoplasm forms a narrow rim around the large, round nucleus. The second type of agranular leukocytes in fish blood are monocytes - large cells whose cytoplasm occupies the main part of the cell. The nucleus of the monocytes is bean-shaped or slightly lobulated, and usually close to the edge of the cell. Polymorphonuclear leukocytes are somewhat smaller than monocytes. The nucleus is lobulated and may be multisegmented. The cytoplasm occupies a considerable part of the cell.

The second group - the granulocyte leukocytes - includes two cell types: neutrophils and eosinophils. Neutrophils are medium-sized cells with an oval nucleus usually located close to the edge of the cell. The cytoplasm of the neutrophils contains numerous small granules which stain a dark-violet with Giemsa-Romanowsky stain. Eosinophils are also medium-sized cells with lobed or bean-shaped nuclei. Their cytoplasm contains numerous granules stained pink with Giemsa-Romanowsky stain.

Except for the spawning period, lymphocytes are the prevailing form of leukocytes in the blood of all species of bony fish. They are especially numerous in the blood of the fry (in the blood of the fry - of - the - year of carps lymphocytes comprise more than 90% of all leukocytes). During spawning the ratio between various forms of leukocytes in the blood of bony fish changes markedly: the number of lymphocytes decreases, and the number of monocytes and morphonuclear leukocytes increases considerably.

The leukocyte formula can also change in various fish diseases. According to E. M. Lyalman, fish suffering from German measles show an increase in the number of neutrophils.

For determination of the leukocyte formula, blood is obtained from the caudal artery (as for counting of leukocytes). A small drop of blood (from the Pasteur pipet) is placed on a carefully washed and defatted slide. The slide should be defatted by immersing it in an alcohol-ether mixture. To prepare a blood film, place the end of a second slide - "spreader slide" - against the surface of the slide with the blood drop at an angle of 45°, and draw it up against the drop of blood which will run across the end of the slide by capillary attraction, filling the angle between the two slides. Push the "spreader slide" back along the other slide, so that the blood drop will follow the "spreader slide". The film should be even, uniform and not too thick.

Type of leukocytes	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	Total number	Percent
Lymphocytes	O	O	O	O	I	P	I	V	G	P	V	P		G	P				G	O	50	25
Monocytes	I	I			I	P		G	I	I	I	I		G	I					I	16	8
Neutrophils	V	O	V	V	O	V	E	V	I	V	O	V	I	G	V	V	V	V	V	V	125	62.5
Eosinophils		I		I		G				I				G	I						8	4
Basophils			I																		I	0.5

FIGURE 3 Table of recording leukocyte types

The films are allowed to dry and then immersed for 3 min in methanol for fixation, then again allowed to dry and stained with Giemsa-Romanowsky stain. The commercially available solution of Giemsa stain is diluted with distilled water of pH 7. 1-2 drops of the stain per 1 ml of distilled water. Distilled water is adjusted to pH 7.0 with the aid of phosphate buffer. The stain should be diluted immediately before use. The diluted stain is drawn up in a pipet and poured over the fixed blood film in a thick layer. The stain is then allowed to act for 20-40 min. Later it is decanted, the remainder rinsed by squirting tap water, and the film is dried by touching it carefully with filter paper.

The stained film is examined under oil immersion. The leukocytes seen near the edge of the film are counted. The slide is moved in a zigzag direction along the edge, then 3-4 fields away from the edge, and then in an opposite direction, etc.

Usually 200 leukocytes are counted. To prevent errors stemming from uneven distribution of leukocytes, leukocytes from four different segments of the film are counted (50 leukocytes per segment). For the sake of convenience all types of leukocytes encountered are recorded on a special table (Fig. 3).

Each leukocyte seen is recorded as a rod in the corresponding column. In each vertical column 10 leukocytes are recorded. The upper figure in the vertical column indicates the number of leukocytes recorded. After all twenty columns have been filled up, the number of leukocytes per horizontal column is determined. By dividing the sum of all leukocytes recorded in each horizontal column by 2, the percentage of each type is obtained.

## SEDIMENTATION RATE (BSR) IN FISH FRY

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To obtain reproducible and comparable results of the erythrocyte sedimentation rate in fry of healthy and diseased fish with a small blood volume, we have somewhat modified the widely accepted method for determining the erythrocyte sedimentation rate in fish (Golodets, 1955). The sedimentation of erythrocytes is carried out in Panchenkov's apparatus. This method requires a capillary tube whose walls are moistened with a 5% solution of sodium citrate; sodium citrate solution is then poured into the tube up to the 6 divisions, and then poured out on a watchglass. With the aid of the same capillary tube blood is drawn up to the 24th division of the tube, and then mixed with sodium citrate on the watch glass. Thus the ratio of blood to sodium citrate is 4 : 1 (as in the original method) but the total amount of blood and sodium citrate in our modified method is considerably smaller. The blood-sodium citrate mixture is drawn into the capillary tube to the mark equal to 25 divisions, i.e. one fourth of that used in the original test. The capillary tube is placed on a rack for one hour, and the volume of packed erythrocytes is then recorded. This method yields satisfactory BSR values.

The BSR values obtained by this method are somewhat different from those obtained by the original method. In order to determine these variations, we examined the blood of a commercial carp by means of the original and modified methods. We determined the BSR in 40 carps at the age of 17 months. Since the variations in values are apparently due to the change in height of the column of citrated blood in the capillary tube, we studied the BSR as a function of the height of the blood column in the tube. Blood was mixed with sodium citrate in proportions used in the original method. The first capillary tube contained citrated blood to the mark 100 (the original method); the second capillary tube contained citrated blood to the mark 50, the third tube, to the 25 mark, and the fourth capillary tube, to the 12.5 mark. All four capillary tubes were placed in a rack, and one hour later the point on the scale which the erythrocytes had reached was recorded. For each blood sample (carp) four BSR values were obtained. The average values of the BSR from 40 determinations were as follows: with the original method: 3.3 mm/hour; with the modified method: 3.6, 4.5 and 4.5 mm/hour, respectively.

The BSR values obtained by the method proposed by us thus differed somewhat from those obtained with the original method. Our method is recommended for cases in which the BSR of small, healthy and diseased fish with a small total blood volume have to be compared.

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## BLOOD GROUPS OF FISH AND METHODS OF THEIR DETERMINATION

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Blood groups were first discovered in human beings. The classification is based on the presence or absence of two separate agglutinins ( $\alpha$  and  $\beta$ ) in the serum and the corresponding agglutinogens (A and B) in the erythrocytes. Human serum containing agglutinin  $\alpha$  is capable of agglutinating erythrocytes of another person containing agglutinogen A. Erythrocytes containing agglutinogen B are agglutinated by serum containing agglutinin  $\beta$ . Blood groups are thus determined by means of agglutination reaction of erythrocytes - hemagglutination.

A thorough knowledge of human blood groups is necessary for blood transfusion. In animals, including fish, blood groups are studied mainly to establish the relation between different animals.

In the majority of fish examined, no isoagglutinins could be detected, and blood groups were revealed by means of immune sera.

For the preparation of immune sera, experimental animals were given intravenous, intraabdominal or subcutaneous injections of fish erythrocytes (antigen). After a certain time agglutinins (antibodies) capable of agglutinating fish erythrocytes appeared in the blood of the immunized animals. Quite good results were obtained by using the method of isoimmunization, in which some fish were immunized against erythrocytes of other fish of the same species.

### DETERMINATION OF BLOOD GROUPS IN FISH BY HEMAGGLUTINATION

For blood typing by the hemagglutination method two reagents are necessary: 1. Fresh erythrocytes of the fish species in question, and 2. Hemagglutinating serum.

**Obtaining and preparation of erythrocytes.** Blood is taken by means of a sterile Pasteur pipet\*. Blood is obtained from the heart, gills or caudal artery and placed in a sterile centrifuge tube. For clotting, the blood is kept in a thermostat at  $+25^{\circ}\text{C}$  for 30 min. The serum is separated from erythrocytes by centrifugation at 1500 rpm for 5 min. Prior to centrifugation the clot is separated from the tube with a stirring glass rod. The serum is discarded or used as a reagent if it contains isoagglutinins. The erythrocytes which sediment during centrifugation are washed thrice with 10 ml portions of 0.9% physiological solution of sodium chloride.

\* Glassware is sterilized in dry heat for two hours at  $180^{\circ}\text{C}$ .

**Obtaining of hemagglutinating serum** Preliminary experiments are designed to ascertain whether fish serum is capable of agglutinating erythrocytes obtained from fish of the same species. The content of isoagglutinins in fish serum is usually judged on the basis of not less than 200 cross-reactions. Erythrocytes are mixed with fish sera on small porcelain slides with wells. If no agglutination is observed, blood typing will have to be done with the aid of immune sera.

Immune serum is prepared in the following way. Experimental animals are given repeated intravenous, intraabdominal or subcutaneous injections of fresh, washed fish erythrocytes. The injections are given several days apart. There are numerous immunization schedules, but there is no universal scheme, since it depends on the individual properties of the animals to be immunized, on the nature of the antigen (fish erythrocytes in this case), the dose of antigen, etc. Several days after the last injection, the animal is bled (trial bleeding), and the blood is tested for antibody titer. When the method of isoimmunization is used, hemagglutinins are produced in the fish. In other respects, isoimmunization does not differ from the immunization.

Before the use of immune serum in the reaction, it must first be subjected to absorption with the erythrocytes examined. For this purpose the serum (in a test tube 8 mm in diameter) is mixed with a certain amount of fresh washed fish erythrocytes. The absorbed serum is then separated from the absorbent by centrifugation. The serum is then used in the reaction with the erythrocytes which served as absorbent. When the reaction is positive, absorption is repeated. The absorbed serum is then tested with the aid of the erythrocytes from the given fish species.

**Hemagglutination reaction** The reaction between hemagglutinating serum and washed fish erythrocytes is best carried out on flat porcelain slides. Two drops of serum are mixed with a small quantity of erythrocytes. The necessary amount of erythrocytes is easily discharged on the porcelain slide by touching the surface of the slide with a Pasteur pipet filled with suspension of washed erythrocytes. Controls consisting of the erythrocytes studied mixed with saline should be run at the same time. Positive agglutination can be considered as such only in cases when the controls (erythrocytes with saline) are not agglutinated. The extent of agglutination is judged by inspection with the naked eye, followed by inspection with the aid of a magnifying glass and under the microscope.

**Equipment, glassware, reagents and materials** Equipment: centrifuge, refrigerator, thermostat, microscope, magnifying glass, racks for test tubes, scissors, scalpel, syringes, alcohol and electric burners.

Glassware: porcelain slides, narrow test tubes (8 mm in diameter), Pasteur pipets, graduated 1 ml pipets, centrifuge tubes.

Reagents and materials: sodium chloride, distilled water, ethanol, cotton wool and gauze.

## IMMUNOBIOLOGICAL REACTIONS

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### DETERMINATION OF PHAGOCYTIZING CAPACITY OF BLOOD LEUKOCYTES

One of the main functions of blood leukocytes is their phagocytizing activity. These phagocytes, as I. I. Mechnikov has shown, engulf pathogenic microorganisms and foreign particles which have found their way into the organism. The microorganisms engulfed are subsequently digested by the enzymes present in the cytoplasm of the phagocytes, while the non-living engulfed particles are carried away by leukocytes and are removed from the organism together with other body excretions. The phagocytizing function is one of the most important factors in the resistance of the organism to diseases; hence, the determination of the phagocytizing capacity of blood leukocytes is one of the criteria of resistance of the organism to various diseases.

For the determination of the phagocytizing activity of blood leukocytes, bacterial suspensions or suspensions of fine carmine granules may be employed. If bacterial suspensions are employed, the method of choice is that of Platonov as modified by G. G. Golodets. According to this method, the blood is obtained from the caudal artery of the fish after cutting off the tail, or according to the method described on p. 12 (for obtaining of blood for counting of leukocytes). Blood is drawn into a diluting pipet, used for counting human leukocytes to the mark 1 and then physiological solution of sodium chloride (0.65% NaCl) is drawn up to the mark 11. The content of the diluting pipet is transferred into a short glass test tube with a tapering end (see figure). The diluting pipet is filled with another portion of physiological sodium chloride solution to the mark 11 and squirted into the test tube with blood. With the aid of the same diluting pipet an equal volume of bacterial suspensions in saline, containing approximately  $10^4$  cells/ $1\text{ ml}^3$ , is added to the test tube.



Test tube for experiments on phagocytosis



The content of the test tube is carefully mixed and incubated at 20°C for 25 min. To prevent sedimentation of blood cells, the content of the test tube should be mixed every 10 min (by rapidly rotating the test tube in the hands in an inclined position). At the end of incubation (after 25 min) phagocytosis is arrested by adding 1 - 2 drops of potassium arsenate (Fowler's solution), or one drop of a 1% solution of osmic acid, and the test tube is centrifuged at approximately 1000 rpm (not more) for 3 min. Blood cells are sedimented in the tapering part of the test tube; with this method the leukocytes appear in the upper layer of the sedimented cells.

The transparent supernatant is drawn off by means of a pipet, and the leukocytes present in the upper layer of the sediment are removed by means of the same pipet and transferred to a slide for the preparation of a film. During the film preparation care should be taken not to damage the leukocytes by the spreader slide. The film is allowed to dry, fixed for three minutes in methanol, and stained with Giemsa. In preparations stained by this method, the bacteria engulfed by the phagocytes are clearly seen in the cytoplasm of the latter. The phagocytized bacteria are counted under a microscope with an oil immersion objective. The number of bacteria encountered in 200 leukocytes is recorded. The counting is done in four different segments of the film (50 leukocytes in each segment are counted). For the sake of convenience a table with 200 squares is drawn. Each square corresponds to one leukocyte. The number of bacteria encountered in each leukocyte is recorded in each square. If the leukocyte in question did not contain bacteria, the corresponding square was marked with 0.

The method for determining the phagocytizing activity of leukocytes with the aid of bacterial suspensions suffers from the drawback that the ability of leukocytes to engulf bacteria can be affected not only by the changes in properties of the phagocytes themselves, but also by changes in the properties of the bacterial cultures used (depending, among other things, on the age of the culture, etc). In addition, certain bacterial species produce toxic substances which inhibit phagocytosis.

These drawbacks can be alleviated by using the method of study on phagocytosis with the aid of a suspension of carmine granules. For studies on phagocytosis by means of this method one must first prepare a suspension of carmine granules. To this end 1 g of carmine is carefully ground in an agate mortar for 15 min. The carefully ground carmine is rinsed from the mortar with saline and into a 200 ml volumetric flask. The volume is made up to 150 - 200 ml with saline. The carmine suspensions is shaken and left until all carmine granules have precipitated. Later, the red-colored supernatant is drawn off and the volumetric flask is again filled to volume with another portion of saline. The carmine granules are allowed to settle, the supernatant is again drawn off, and the entire procedure is repeated until the supernatant remains colorless. This procedure aims at removing very fine (non-sedimentable) colloidal particles of carmine from the solution.

Suspensions of carmine granules prepared in this way (provided the elementary rules of sterility have been observed) may be stored for years. For the experiments on phagocytosis, carmine suspensions should be shaken for several hours before the experiment. Several hours later carmine granules commence to settle, and a transparent supernatant is clearly demarcated from the layer of the precipitating carmine granules. In order to obtain carmine particles of the desired size the tip of a Pasteur pipet is

introduced to a depth of about 2 cm below the upper border of the layer of precipitating earmine particles, and the required amount of carmine suspension is carefully drawn into the pipet. The suspension obtained in this way consists of carmine particles 2-4  $\mu$  in size. The number of carmine particles per 1 mm<sup>3</sup> of suspension can be determined by counting them in a counting chamber used for counting of blood cells. The carmine suspension is then diluted with saline to the required concentration. The suspension most suitable for studies on phagocytosis should contain 15,000-20,000 carmine granules per mm<sup>3</sup>.

Having prepared a earmine suspension of the desired concentration, 0.6 ml of the suspension are transferred into a test tube with a tapering end (similar to that used in studies on phagocytosis with the aid of bacterial suspensions), and incubated for 30 min at the temperature of the experiment. Later 20 mm<sup>3</sup> of blood obtained from the caudal artery of the fish under study are added to the test tube containing the earmine suspension. The carmine-blood mixture is kept at a constant temperature for 90 min (at 20°C in studies with fish of our latitudes). In order to prevent sedimentation of blood cells, the content of the test tube should be shaken (the test tubes are best placed in Warren's shaker with a rate of 2-3 oscillations per minute). If the above shakers are not available one can mix the content of the test tube by hand-rotating them every 15 min.

At the end of the 90 min of incubation, phagocytosis is arrested by adding 1-2 drops of Fowler's solution. The test tube is then centrifuged and the upper layer of the sedimented cells is used for preparing films which are fixed in the same way as described above in studies on phagocytosis with the aid of bacterial suspensions. The fixed film is stained for 30-40 sec with a 1% aqueous solution of methylene blue, and the stain excess is then rapidly removed by immersing the film in water. The film is dried by carefully touching it with filter paper, and examined under an oil immersion objective. The leukocytes are stained blue, their nuclei are readily seen, the carmine granules present in the cell cytoplasm are stained a brownish-red. Counting of carmine granules engulfed by leukocytes is done in the same way as in the phagocytized bacterial cells described above.

#### Obtaining of hemolysins in fish blood

It was shown by I.I. Mechnikov that when fish were given subcutaneous injections of erythrocytes from other animal species, they developed after some time specific antibodies capable of agglutinating and lysing erythrocytes of the species whose red blood cells were used for immunization.

For the preparation of hemolytic sera erythrocytes from rams or other animals can be used. The erythrocytes are washed three times with saline (0.9% solution of NaCl) and diluted with saline 1:1. Fish are given subcutaneous injections of washed red blood cells — 1 ml of erythrocyte suspension per 100 g body weight of the fish. The fish are given 5-6 subcutaneous injections, 3 days apart. Seven-eight days after the last injection the caudal fin of the fish is cut off and the blood coming out from the caudal artery is collected in a test tube. The test tube is then placed in a thermostat at 25°C for clotting. After one hour of incubation the transparent serum is separated from the clot by centrifugation and collected with a pipet.

For determination of the titer of hemolysins, the erythrocytes from a donor animal (which served for fish immunization) are washed with saline

and resuspended in the latter, to obtain a 5% erythrocyte suspension, 0.5 mm of this suspension are added to a series of 5-10 test tubes with immune serum in varying dilutions. Serum is diluted with saline in the following ratios 1:1, 1:2.5, 1:5, 1:10, 1:20, etc. The tubes are incubated in a thermostat at 25°C for 1 hr, by determining the tubes in which hemolysis took place the presence and titer of hemolysins in the serum can be ascertained

#### PRECIPITATION

The precipitating activity of immune sera can be detected on mixing them with a solution of foreign protein (precipitinogen) which was used in the immunization of the animal (whose serum is tested for the presence of precipitins). As a result of mixing, a turbidity first appears and a precipitate is then formed. When the immune serum is layered over the transparent solution of precipitinogen a turbid ring appears at the interphase between the serum and antigen.

Antibodies which appear in the serum of the immune animal are called precipitins and are not strictly specific. The immune serum may give positive reactions of precipitation with proteins of other closely related animal species. Thus, for example, serum of rabbits immunized against human proteins gives positive reaction with proteins of monkeys, whereby the precipitation reaction is more distinct the more closely evolutionally related the species of monkeys is to man. Precipitation has been used in biology to establish species or race relationships between various animals. Precipitation reaction is used in ichthyological practice to establish evolutionary relationships between different fish species. Fish serum, obtained in the same way as that for hemolysin reaction, is used as precipitinogen. When the study is carried out on very small fish, their muscles can be used, after freeing them completely from other tissues. The muscle tissue used for this purpose is carefully ground to pulp in a sterile glass or porcelain mortar. Glass or heat-sterilized sand is added to the mortar for better grinding. The carefully ground pulp is diluted 1:1-1:5 with Ringer-Locke-alcohol solution and left for one day on ice. The Ringer-Locke-alcohol solution has the following composition: NaCl, 9 g, KCl, 0.2 g, CaCl<sub>2</sub>, 0.2 g, NaHCO<sub>3</sub>, 0.2 g, glucose, 1 g, absolute alcohol, 150 g, distilled water, 850 g. After one day of extraction, the extracts are filtered off (through cotton or filter paper) and sterilized by passing through a filter with infusorial earth. The sterile extracts are then dispensed in sterile test tubes with ground stoppers and kept in the cold. Precipitinogens prepared in this way can be stored for several months.

In this way antigens are prepared for immunization and for precipitation reactions.

To obtain antibodies rabbits are given intravenous or intraabdominal injections of the above antigen (1 ml of the antigen per 1 kg of body weight of rabbit). Four injections, 5-6 days apart, are given. Eight days after the fourth injection, trial bleeding is performed. The blood obtained is allowed to clot, and the transparent serum which separates is drawn off and tested for the presence of precipitins. Prior to bleeding, rabbits are kept for 12-16 hours on hay and water.

The precipitating activity of the serum is determined in the following way: 1 ml of the clear solution of precipitinogen is added to a series of small serological test tubes. Then to each of these test tubes (except the first one which serves as control) increasing amounts of the immune serum are added. It may be used undiluted or diluted (10-fold and 100-fold dilutions in saline (depending on serum activity)). The test tubes are shaken and kept in a thermostat at 37°C; after 2 hrs, it is taken out and the mixture. Positive reaction can be ascertained by the

appearance in the transparent liquid in the test tube of flocculi which settle at the bottom of the test tube in the form of a loose precipitate. This method is not always suitable. Precipitation reaction is characterized by the so-called optimal zone phenomenon: the intensity of the reaction is not always proportional to the concentration of antibody and antigen used, but is more pronounced when the latter are mixed in optimal proportions. For this reason the ring test is more suitable. In this method antigen is layered over the immune serum, so that optimal concentrations are created at the interphase between these two liquids.

For the ring test reaction, undiluted immune serum (or diluted 1:1 with physiological - 0.9% - solution of sodium chloride) is used. The antigen is diluted with saline in the following proportions. 1:10, 1:100, 1:1000 etc. Serum is added to 7 serological (narrow) test tubes; 0.2 ml of serum are placed in each test tube with a Pasteur pipet, so as not to moisten the walls of the test tube. A similar volume of antigen is then allowed to flow along the wall to form a layer over the serum. The same pipet may be used for all antigen dilutions, provided the highest antigen dilutions are taken first. In the first, control, test tube the immune serum is covered with saline (instead of antigen). The solutions should not mix at the interphase.

When the test tubes are kept for 30 - 60 min at room temperature the positive reaction is manifest in the appearance of a white dense ring at the interphase. The more potent the serum the thicker the ring. Observation is continued for 2 hours. The reaction is best read in a good light against a dark background. The minimal concentration of antigen capable of giving a ring is recorded. In order to be able to determine this concentration with greater accuracy, 1:2 dilutions of the antigen close to the concentration which causes the appearance of the ring may be used (instead of 1:10 dilutions used in the first ring test) (for example dilution 1:100, 1:200, 1:300 etc).

# ELECTROCARDIOGRAM RECORDING IN LONG-TERM EXPERIMENTS

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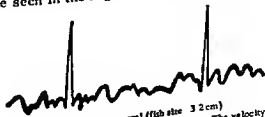
In order to obtain ECG's of fish leading a free life in an aquarium, a battery-fed amplifier of biocurrents and the EO-7 cathode oscillograph must be used. Bits of small fish-hooks soldered to the ends of multiple light conductors may serve as tapping electrodes. The fish-hook itself and the point at which it is soldered to the conductor is carefully insulated by polystyrene, so that only the inner surface of the bent part of the hook remains non-insulated. At this point the contact between the fish body and the hook is made. The ends of the conductors pass through a cork floating on the water. Further connection of electrodes to the input of the amplifier is done by shielded conductors.

For the experiments, electrodes are inserted into the body of the fish, one at the anterior part of the back and the other on any part of its abdomen. The electrodes are fixed to muscles with the aid of thin silk. The form of the ECG depends on the location of the abdominal electrode. When the fish seems accustomed to the electrodes, it is placed in an aquarium provided with an iron or glass bottom. In the latter case the aquarium should be placed on a tin sheet plate.

Studies on the effect of electrodes on fish behavior have shown that fish with fixed electrodes swim quietly for hours at a time, take food and do not differ in any respect from all other fish present in the same aquarium.

In order to record ECG's, the batteries, feeding hoses and the water of the aquarium should be carefully screened. To this end a conductor is soldered to the bottom of the aquarium, or a plate of galvanized iron 5 cm long and 10 cm wide connected to the body of the amplifier is placed into the aquarium.

The ECG is recorded on an electrocardiograph or on a train oscillograph. In the latter case the lead should contain an extreme amplifier. An example of such recording on electrocardiogram of gourami (*Dsiphronemus goramy*) can be seen in the Figure.



Electrocardiogram of gourami (fish size 3.2 cm)  
Graduation 1 mv. = 40mm of ray deviation. The velocity with which the tape was moved was 60mm/sec

The method described above is a convenient one since it permits simultaneous observations on the respiratory movements of the fish. To this end, the abdominal electrode is inserted into the body over the skin layers in the region of the heart. In such a case the electrocardiogram shows fluctuations of the potential in the respiratory rhythm superimposed by prong complexes.

# METHODS OF STUDY OF RESPIRATION IN FISH

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CHEMICAL AND PHYSICAL METHODS FOR WATER ANALYSIS AND METHODS FOR PREPARING  
WATER WITH LOW OXYGEN PARTIAL PRESSURE

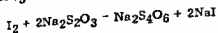
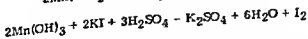
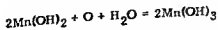
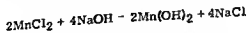
## Determination of Oxygen Dissolved in Water

### Determination of oxygen by the method of Winkler

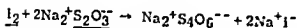
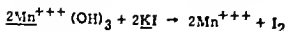
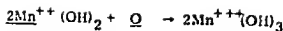
The amount of oxygen dissolved in water is determined by the method proposed by Winkler. Since in the majority of experiments on oxygen uptake by fish purified water is employed, we shall here describe the original method without dwelling on those modifications which must be introduced for analyses of water with increased oxidizing or reducing properties.

The method is based on three successive oxidation-reduction reactions. In the course of the first reaction the oxygen dissolved in water becomes fixed, in the course of the second reaction, free iodine equivalent to the amount of oxygen fixed is liberated, and in the course of the third reaction, free iodine is titrated with a standard sodium hyposulphite solution. The water oxygen content is calculated from the amount of sodium hyposulphite used in the titration.

The method of oxygen determination makes use of the following chemical reactions



These reactions may be written in ionic form, in order to emphasize their oxidation-reduction nature (additional reaction components are omitted and the elements in which the main reactions take place are underlined)



According to the nature of the reactions one can expect that all admixtures endowed with oxidation-reduction properties which may be present in water in a suspended or dissolved state will interfere with the above reactions and lead to errors in determination of the oxygen content.

Some explanations: The first and the second reactions represent the fixation of oxygen dissolved in water. The third reaction involves the dissolution of the precipitate with the liberation of free iodine, while the fourth reaction represents the titration of free iodine with sodium hyposulphite. For convenience, KI is added to the NaOH solution, and this mixture is poured into the pyknometer during fixation. The reagents  $\text{MnCl}_2$  and  $\text{NaOH} + \text{KI}$  are frequently called Winkler's reagents.  $\text{Mn}(\text{OH})_2$  (manganous hydroxide) is a white precipitate,  $\text{Mn}(\text{OH})_3$  (manganic hydroxide) is a brown precipitate. The color intensity is proportional to the amount of oxygen fixed. The difference between the color of the two precipitates forms the principle of the method for the colorimetric determination of oxygen proposed by I. N. Arnold some 50 years ago (for fish-breeding purposes).

The fixation of free oxygen dissolved in water by means of manganous hydroxide which is transformed into manganic hydroxide is sufficiently rapid. With the decrease in oxygen concentration, however, the rate of the reaction decreases and becomes very slow in the presence of oxygen traces. Besides, the precipitate of  $\text{Mn}(\text{OH})_3$  formed is a very loose one and settles slowly on the bottom of the container. Since the subsequent reaction takes place in an acid pH, one must wait a certain time to allow a quantitative precipitation of  $\text{Mn}(\text{OH})_3$ , so that after adding acid the solution displaced from the pyknometer will not contain the fine precipitate of  $\text{Mn}(\text{OH})_3$ . As a rule, 40-60 min suffice for the precipitate of  $\text{Mn}(\text{OH})_3$  to settle quantitatively on the bottom, and for the upper layer of the solution to clear completely. After this time, the precipitate must be dissolved and the free iodine titrated.

The sample with fixed oxygen may be kept in the dark for 15 days. Longer storage should be avoided, and it is recommended to carry out the analysis one or two days after sampling.

The third reaction takes place in acid pH. There are indications in our literature that the precipitate may be dissolved by concentrated hydrochloric acid.  $\text{HCl}$  may, however, cause a considerable error if it contains free  $\text{Cl}_2$  or if the sample to be analyzed contains iron. In the latter case  $\text{FeCl}_3$  is formed, which reacts with  $\text{KI}$  with the liberation of free iodine which is subsequently titrated with sodium hyposulphite. It is better to acidify water with orthophosphoric or sulfuric acids. The use of these acids does not lead to the errors mentioned above.

After the precipitate has been dissolved the sample should not be left for a long time without titration, because free iodine, being an oxidizing agent, will oxidize all organic substances which may be present in the water sample.



The fourth reaction is a rapid one. It must be remembered that free iodine is volatile, and the iodine dissolved in the sample may partly evolve on standing in an open titration vessel. The volatility of iodine increases with temperature rise. Hence, the sample transferred from the pyknometer into a conical flask should be titrated immediately. At first sodium hyposulphite is added without indicator, until the solution is almost completely decolorized. When the sample is almost colorless, starch solution (indicator for iodine) is added. The sample assumes a blue or weakly violet color, depending on  $I_2$  concentration and on the color of the water sample. Further titration should be done carefully, adding sodium hyposulphite drop by drop to full decolorization of the sample. When the sample is decolorized, the aliquot of the sample is returned to the pyknometer to rinse it from the remaining free  $I_2$  and the solution is again decolorized by adding sodium hyposulphite drop by drop. The amount of sodium hyposulphite used up for the titration is recorded and the water oxygen content calculated.

If the water sample contains a high concentration of organic substances or products of metabolism, the permanganate modification should be employed, this consists of preliminary oxidation of the sample by permanganate in acid medium.

## Determination

**Glassware and reagents** For sampling and oxygen fixation pyknometers or flasks with ground stoppers are necessary. The stoppers should have a taper, so that during closure of the pyknometer no air bubbles will remain. Before the determination, the pyknometers should be calibrated, 50 - 70 ml pyknometers are preferable. If the water sample is smaller, 3-5 ml pyknometers may be used. The determination should be carried out very carefully.

Calibration consists of the following: the pyknometers are carefully washed, dried, numbered and weighed (tare) with an accuracy to 0.01 g (the volume of the pyknometer is 50 - 70 ml). The pyknometers are then filled with distilled water at room temperature, the water on the outside is removed by means of filter paper and the pyknometers are reweighed with the same accuracy. The difference in weight shows the weight of water in the pyknometer. To calculate the volume of water the weight obtained is multiplied by the corresponding coefficient for the given temperature of water. The product is the volume of the pyknometer. Coefficients for various temperatures may be found in the handbook "Chemist's Guide" (Sputnik Khimika) (Vol. 1).

Six Mohr's pipets (or graduated pipets) are needed—four 1 ml pipets, one 3 ml pipet and one 20 ml pipet (graduated). Two titration burets (better with milky band and graduations to 0.05 or 0.1 ml and a total volume of 25 ml). Five 150 - 200 ml conical flasks are used for titration.

The solutions should be prepared from chemically pure reagents (analytical grade). Special attention should be paid to the purity of the KI reagent. This reagent should not only be chemically pure for analysis, but completely free of molecular iodine which appears during photo-decomposition of KI, or during careless storing of KI. If KI is contaminated (the reagent assumes a weakly yellowish color) it should be dried at 110°C to remove  $I_2$  (the reagent turns white).

1.  $\text{MnCl}_2$ , 32% solution, and 2  $\text{NaOH}$ , 32% solution +  $\text{KI}$ , 10% solution, these should be stored in the dark.  $\text{KOH}$  may be used instead of  $\text{NaOH}$ , but in 1.5 times greater quantity

3.  $\text{H}_2\text{SO}_4$  1 l, or  $\text{H}_3\text{PO}_4$  concentrated (85%)

4. 0.2% starch solution (should be freshly prepared).

5.  $\text{Na}_2\text{S}_2\text{O}_3$ , 0.01 or 0.002 N (when small pyknometers are used).

Sodium hyposulphite of approximately known normality is first prepared and then standardized by titration with a standard solution of potassium dichromate  $\text{K}_2\text{Cr}_2\text{O}_7$ .

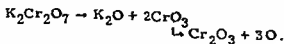
Preparation and standardization of sodium hyposulphite solution. Solution of sodium hyposulphite of desired normality is prepared in the following way: distilled water is boiled to kill any bacteria present and then cooled. A weighed sample of  $\text{Na}_2\text{S}_2\text{O}_3$  is dissolved in distilled water prepared in the way described above. It should be remembered that in this reaction hyposulphite acts as a reducing agent, and its equivalent weight is unity, hence its normality is equal to molecular weight:

$$(N = \frac{M \times v}{1}).$$

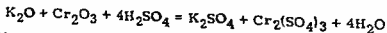
Preparation of 0.01 N solution of  $\text{Na}_2\text{S}_2\text{O}_3$ . Weigh out 2.48 g of the salt and dissolve in water. Make up the volume with distilled water to 1000 ml. To preserve the initial normality of hyposulphite for longer periods of time it is recommended to add a preservative to the solution (a drop of metallic mercury or thymol crystals) and the stopper should be provided with an absorption train containing soda-lime (to absorb  $\text{CO}_2$  from the passing air). The bottle containing the reagent should be wrapped in black paper or stained black (to protect the solution against light). It is desirable for the solution to be kept for 7 - 10 days prior to use (during this period its normality alters greatly).

For standardization of the hyposulphite solution an accurate 0.01 N solution of potassium dichromate is prepared. For this 0.4903 g of very fine crystals of twice recrystallized potassium dichromate ( $\text{K}_2\text{Cr}_2\text{O}_7$ ) are weighed out, dissolved in distilled water, and made up to a volume of 1000 ml. Such a solution may be kept for a long time in a clean bottle.

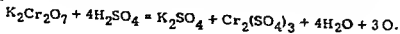
Determination of the normality of sodium hyposulphite solution is based on the following reactions. In acid medium the dichromate decomposes with the liberation of atomic oxygen, which then acts on  $\text{KI}$  and liberates equivalent amounts of free iodine. The latter is titrated with hyposulphite. At the end of the reaction, starch solution is added as an indicator. These reactions can be represented schematically in the following way:



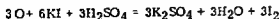
To drive the reaction in the desired direction the medium must be acid, in order to dissolve the  $\text{K}_2\text{O}$  and  $\text{Cr}_2\text{O}_3$  oxides.



net reaction:

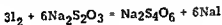


As a result of this oxidation-reduction reaction, three atoms (or six equivalents) of oxygen are liberated. The normality of potassium dichromate is equal to the molecular weight divided by  $(N = \frac{MX_v}{6})$ . This reaction takes place only in the presence of oxygen acceptors. For our purpose KI and hydrogen ions serve as oxygen acceptors, in this respect iodine ions are the donors of excess electrons, while atomic electroneutral oxygen is the acceptor. The reaction takes place according to the following scheme

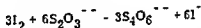


As a result of this electron exchange reaction, iodine ions lose electrons and are converted into molecular iodine, while oxygen atoms acquire electrons and are converted into "ionic" oxygen which immediately combines with hydrogen ions to form water.

The reaction which underlies the titration of free iodine with sodium hyposulphite takes place according to the following formula



or



Ionic iodine does not give blue-violet color in the presence of starch, for this reason the latter is used as indicator for determination of the end point of the reaction.

Standardization of hyposulphite. Into a 100 - 150 ml flask the following reagents are poured in the order indicated: 1 ml KI 15% + 3 ml  $H_2SO_4$  25% + 20 ml 0.01 N  $K_2Cr_2O_7$ . Immediately after adding potassium dichromate to this medium, free iodine begins to appear, giving the solution a dark-brown-yellow color. To prevent the escape of free iodine, the flask should be covered with a watch glass, and left for 3 min, to allow all molecules of potassium dichromate to react with KI. Since  $K_2Cr_2O_7$  serves as the primary standard, exact amounts of potassium dichromate must be taken. For this purpose graduated Mohr's pipets are used.

For the titration of free iodine, hyposulphite is first added rapidly from a buret, and when the titrated solution assumes a weakly-yellow color, it is added drop by drop until the solution has only a barely detectable yellowish tinge. Thereafter, 0.5 ml of 0.2% starch solution is added and the solution is titrated with hyposulphite until the disappearance of the blue color (the color of starch in the presence of free iodine). It must be remembered that  $Cr_2(SO_4)_3$  is formed, giving the solution a weakly green color, thus making the end point determination of the reaction difficult. To obviate this difficulty it is recommended to take one flask with titrated solution as a standard (hyposulphite solution in this flask should be in excess) and the color of all titrated solutions should be matched with this solution. Hyposulphite excess does not change the color of  $Cr_2(SO_4)_3$ . The determination should be carried out at least in triplicate. If the results differ from one another by no more than 0.02 - 0.03 ml of hyposulphite, the average figure is taken and the normality of hyposulphite determined accordingly. For example: exactly 20 ml of 0.01 N solution of  $K_2Cr_2O_7$  were taken. M ml of hyposulphite were used in titration,

the correction will be  $A = \frac{20}{M}$ . This means that the hyposulphite solution is not exactly 0.01N. To calculate to how many ml of exactly 0.01N solution of hyposulphite B ml of hyposulphite used up for the titration of the sample correspond, B should be multiplied by the correction A. The normality of hyposulphite should be checked periodically, since it changes gradually.

**Determination:** Pyknometers are carefully filled with water to be analyzed by means of a syphon. The syphon is placed on the bottom of the pyknometer and water is poured in. The inflowing water ascends relatively slowly and replaces the upper water layers which contain a certain amount of air. The upper water layers should be replaced to one third of the volume of the pyknometer. After this, without closing the pyknometer, 1 ml of each of Winkler's reagents are added. The pipet with the reagent should be placed inside the pyknometer at approximately one third of its height. The reagent should be poured slowly from the pipet. To prevent rapid discharge of the reagents, the tip of the pipet should be as thin as possible. First sodium hydroxide solution with KI is added, this being followed by  $MnCl_2$ . After the two reagents are poured inside the pyknometer, the latter should be stoppered so that no air bubble is left under the stopper. The content of the pyknometer is mixed by gently turning the pyknometer upside down. The precipitate formed is distributed throughout the pyknometer and assumes a color the intensity of which depends on the oxygen concentration. The sample with fixed oxygen is kept in a dark place for 1-2 hours or 1-2 days, depending on the time when the analysis continued. After this, the pyknometer is carefully opened and 1 ml of concentrated  $H_3PO_4$  or  $H_2SO_4$  (1:1) is added. The pipet with acid should be introduced inside the pyknometer for one fourth its height. The acid is carefully poured into the pyknometer, otherwise the precipitate is disturbed and may ascend to the top of the pyknometer and be lost in part during closure of the latter. The pyknometer is closed with the stopper so that no air bubble will remain under it. After mixing the content of the pyknometer, one must wait until the entire sediment has dissolved. If the precipitate dissolves slowly (this occurs when the sample with fixed oxygen has been kept for long time) the sample should be mixed several times.

After the precipitate has completely dissolved, the solution can be titrated. First the normality of the hyposulphite solution is determined. Then the content of the pyknometer is transferred quantitatively into a conical flask and titrated with hyposulphite to produce a hardly detectable yellowish color. After this, 0.5 ml of a 0.2% solution of starch is added and the solution is again carefully titrated with hyposulphite until the blue color disappears completely. Part of the decolorized solution is then poured back into the pyknometer, and after rinsing the latter the solution is again transferred into the conical flask. The solution should assume a pale blue color, and is again titrated with several drops of hyposulphite and discharged from the buret until complete decolorization of the solution.

The final result of the titration is recorded.

**Calculation:** For our purpose the amount of oxygen dissolved in 1 ml of water prior to ( $m_0$ ) and after ( $m_1$ ) the experiment should be determined. The difference between these two values multiplied by the volume of water in which the fish was placed for the experiment will show the total amount of oxygen taken up by the fish, and when divided by weight and time - the relative oxygen uptake.

$$m_0 = \frac{n_0 \times A \times 0.08}{V_0 \times v}; \quad m_1 = \frac{n_1 \times A \times 0.08}{V_1 - v}$$

$$K = \frac{(m_0 - m_1)V}{Px_t}$$

where  $n$ —ml of hyposulphite used for titration of the sample before ( $n_0$ ) and after ( $n_1$ ) the experiments;  $A$ —correction for the normality of hyposulphite; 0.08—the number of mg of oxygen corresponding to 1 ml of 0.01N solution of sodium hyposulphite,  $V_0$  and  $V_1$ —the volumes of pyknometers with water samples prior to and after the experiment;  $v$ —volume of Winkler's reagents added (in our case, 2 ml);  $V$ —volume of water in which the fish was placed for the experiment;  $P$ —weight of the fish;  $t$ —duration of the experiment (in hours);  $K$ —amount of oxygen taken up by the fish, expressed in milligrams of oxygen per 1 g body weight per one hour (mg O<sub>2</sub>/g/hr). If the fish is relatively large, its volume should also be accounted for. In that case not  $V$  but  $V-P$  is taken. If one wishes to express oxygen uptake not in mg but in ml, then the multiplying factor will be 0.0558 instead of 0.08.

If the water sample contains considerable amounts of reducing substance, the permanganate modification ought to be carried out. To the sample to be analyzed 0.3 ml of H<sub>2</sub>SO<sub>4</sub> (1:1) are added; this is followed by adding 0.2 ml of approximately 0.1N KMnO<sub>4</sub>. The pink-colored water is left for 10 min. The pink color should not disappear (indicating KMnO<sub>4</sub> excess); 10 min later, 0.2 ml of a 3% solution of K<sub>2</sub>C<sub>2</sub>O<sub>4</sub> are added (to decompose KMnO<sub>4</sub> excess). To the decolorized sample, Winkler's reagents are added (first NaOH + KI and then MnCl<sub>2</sub>). The subsequent procedure is similar to that described above. For calculation one must account for the volume of oxidizing reagents added. In our case it will equal not to 2 ml as in the original method, but to 2.7 ml (0.7 ml added prior to adding Winkler's reagents).

#### OXYGEN DETERMINATION BY MEANS OF POLAROGRAPHIC METHOD

The method of electrochemical analysis proposed by Yaroslav Heyrovsky (1937, 1951, 1958) is known as the polarographic method. First it was used for the analysis of cations, but soon it became widely used in the determination of various substances. Determination of oxygen in water is a particular example of the use of polarographic methods of analysis. The following phenomenon accounts for the method: If direct electric current is passed through an electrolyte, then at a certain EMF positively charged particles on the cathode will accept electrons, become reduced and precipitate; the substances on the anode will lose electrons to the anode and be oxidized. In other words, electrolysis or reduction-oxidation reaction will take place. When instead of one ordinary electrode, the so-called constant electrode, another (self-regenerating) dropping-mercury electrode is used, electrolysis will take place in a very small volume of the solution owing to the small size of the drop. Owing to constant regeneration of the surface (the drops are constantly formed) the dropping-mercury electrode is not subjected to chemical changes during the passage of electric current. This enables one to detect a correlation between the nature and quantity of the reducible substance on the one hand, and the current and EMF on the other hand. For the analysis,

current-voltage curves are plotted. The nature of the substance is shown by the location of the curve on the graph, and its concentration by the height of the curve. Hence from polarization curves (polarograms) obtained with the aid of polarographs, one can determine the concentration and nature of the reducible compound in question (Fig. 1).

This method is sufficiently simple and satisfactory for the analysis of numerous electrolytes and nonelectrolytes capable of undergoing oxidation and reduction on the surface of the electrode.

Let us now discuss the theoretical aspect of this method, and the conditions under which the analysis should be carried out so as to obtain reproducible results.

As has already been mentioned, the passage of current brings about the precipitation of ions. Different ions precipitate at different EMF, i.e., different ions have their specific reduction (or oxidation) potentials which serve as the basis for their qualitative determinations. In a general form this can be expressed in the following way:



Ions with positive charges  $n(n +)$  may undergo reduction and become converted into electrically neutral ions ( $M^0$ ) by receiving a certain number of electrons ( $ne$ ). For this reaction to proceed, one must apply a certain EMF,  $E$ , which consists of decomposition voltage and voltage necessary to overcome the resistance of the solution  $IR$ . The decomposition voltage is equal to the difference between the potentials (potential peaks on the electrode-electrolyte boundary) or the anode and cathode ( $E - E$ )

$$E = (E_a - E_k) + IR.$$

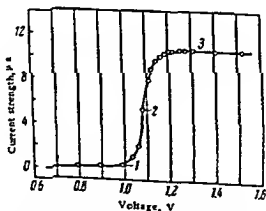


FIGURE 1. Polarization curve (polarogram)  
1 - decomposition voltage; 2 - half-wave potential, 3 - plateau - diffusion current

The strength of the current  $I$  passing through the electrolyte is negligibly small (not more than  $10^{-6}$  A); since the concentration of the reducible ion is low, and the resistance of the electrolyte does not exceed  $10^3$  ohms, the product  $IR$  is negligibly small and can be ignored.

In polarographic techniques the surface of the anode is considerably larger than that of the cathode. Owing to this, the cathode becomes polarized in the course of electrolysis, and its potential changes, while the anode (with a considerably larger surface) is polarized to a slight extent only and its potential remains practically constant. Arbitrarily the potential of the anode is taken as zero ( $E_a = 0$ ); the EMF applied under these conditions is equal to the electrode potential of the cathode

$$E = -E_k.$$

When the dropping-mercury electrode is used (as a cathode) the ion discharged on the cathode forms amalgams. The magnitude of the cathode potential as a function of concentration is expressed by the equation of Nernst:

$$E = -\frac{RT}{nF} \ln \frac{KC}{[Me^{n+}]}$$

where  $R$  - gas constant;  $T$  - absolute temperature,  $n$  - the valence of reducible ion;  $F$  - number of electrons equal to 96,500 Coulombs,  $K$  - constant of the given metal;  $C$  - metal concentration in the amalgam;  $[Me^{n+}]$  - metal ion concentration in the solution.

The amount of the metal deposited is proportional to the current strength  $I$ , i.e.  $C = K_1 I$ .

By substituting this expression in the former equation we shall obtain:

$$E = -\frac{RT}{nF} \ln \frac{K \times K_1 I}{[Me^{n+}]} \text{ or } E = -\frac{RT}{nF} \ln \frac{K_2 I}{[Me^{n+}]},$$

This equation solved for current strength gives:

$$I = \frac{[Me^{n+}]}{K_2} \times \frac{-E \cdot F}{\frac{RT}{n}}$$

where  $e$  is the base of natural logarithms.

This equation of the current-voltage curve is expressed by an exponential function and has an S-shaped form with a bend at the beginning and end.

Molecular oxygen is capable of being reduced on the dropping-mercury electrode and to give rise to two wave peaks. The first wave appears at the beginning of the polarographic curve and the other at about 0.8 volts.

The general nature of curves obtained during polarography can be expressed in the form of a polarogram (current-voltage curve) of some metals (Fig. 2).

Any substance determined by means of polarography is expressed in some segment of the current-voltage curve - this is the basis of the qualitative analysis. Each substance is characterized by its deposition potential.

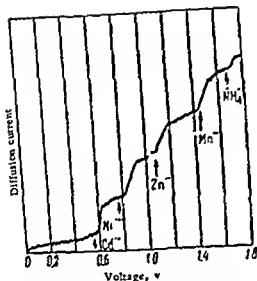


FIGURE 2. Current-voltage curve for several substances simultaneously present in the solution

Concentrations are determined by the height of the wave. Polarograms of one and the same substance taken in two concentrations is shown in the current-voltage curve in Fig. 3.

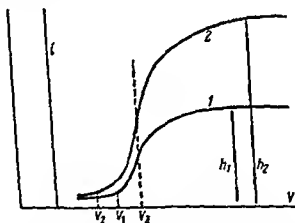


FIGURE 3. Deposition potential (deposition of the substance on the electrode) as a function of concentration of the substance in the solution (two concentrations 1 and 2)

$V_1$  and  $V_2$  - deposition potentials at various concentrations,  $V_3$  - half-wave potential,  $h_1$  and  $h_2$  - height of waves at various concentrations

The concentration of the reducible substance to be analyzed is determined by the height of the wave. It can be seen from Figure 3 that deposition potentials of one and the same substance vary, depending on its concentration in the solution. Hence the determination of the substance (qualitative analysis)



by its deposition potential ( $V_1$  and  $V_2$ ) is rendered difficult. The half-wave potential  $V_3$ , which depends on the nature of the substance and not on its concentration, is more constant. Half-wave potentials of many elements are compiled in tablea (Lur'e, 1947 and Heyrovsky, 1951).

Concentration is judged from the height of the wave. With increase in voltage on the cathode to the value of the reduction potential of the reducible substance (for example of oxygen) on the electrode surface (for example, on the dropping-mercury electrode) the substance is reduced. As a result the amount of reducible substance at the electrode surface decreases, and other particles of this substance will diffuse from the bulk of the solution towards the electrode. A stationary state is attained between the amount of the substance undergoing reduction on the electrode and that diffusing towards the electrode. During such a stationary state the current remains constant. The rate of diffusion of substances to the electrode depends on its concentration, hence the current also depends on the concentration of the reducible substance. For each concentration of reducible substances there is a steady state with its constant current, such current being called diffusion or maximum current. In other words, the concentration of the reducible substance can be determined from the height of the wave or from the magnitude of the diffusion current.

Particles of the reducible substance reach the cathode not only by diffusion, but also by migrating in the electric field created by the electrode. Hence, the maximum current consists of diffusion current (discharge of particles diffusing towards the electrode) and migration current (discharge of migrating particles). Since only the diffusion current is proportional to the concentration of the electroreducible ions, for quantitative analysis it is essential to eliminate the migration current. To this end a "background" is introduced into the solution to be analyzed. The "background" consists of an indifferent electrolyte (usually 0.01N or 0.001N solution of KCl). Ions of the indifferent electrolyte are reduced at a potential which is considerably lower than that needed for the reduction of the reducible substance to be analyzed, while the ion concentration of the indifferent electrolyte exceeds many times that of the substance to be analyzed. All positively charged particles will move in the electric field towards the cathode. Since the concentration of substance to be analyzed is considerably lower than that of the "background", the number of particles of the former moving towards the electrode under the influence of electrical field is negligible as compared with the number of diffusing particles. Only particles which diffuse to the cathode will be reduced in practice. Hence the magnitude of maximum current will be equal to the diffusion current.

Ilkovic proposed the following formula expressing the dependence of the magnitude of diffusion current on the concentration of the electroreducible ions (for uncharged reducible particles):

$$i = 0.627 \times n \times F \times D^{1/2} \times C \times m^{2/3} \times t^{1/6},$$

where  $i$  — diffusion current in amperes; 0.627 — sum of numerical constants,  $n$  — valence;  $F$  — Faraday number (96500),  $D$  — diffusion coefficient (sqcm/aec);  $C$  — concentration, mole/ml;  $m$  — mass of mercury dropping from the capillary in time unit, mg/sec;  $t$  — life of the drop (dropping period), in sec.

To determine the concentration, let us write this equation in relation to C.

$$C = \frac{i}{0.627 \times n \times F \times D^{1/2} \times m^{2/3} \times t^{1/6}}$$

In this formula, the most difficult to determine is the diffusion coefficient. This circumstance hampers the wide use of this equation in analytical practice.

With the increase in concentration of the diffusing substance, the diffusion coefficient continuously decreases. This is more pronounced with neutral molecules than with electrolytes.

The diffusion coefficient is not only difficult to determine, but does not represent a characteristic property of diffusing molecules or ions. The diffusion coefficient also depends on the properties of the solvent. In other words, diffusion is a characteristic property of the entire system.

While deriving the Ilkovic formula, it has been assumed that around the growing drop of mercury in the dropping-mercury electrode there is only a symmetrical spherical diffusion, and that in the presence of a constant amount of electrolyte the maximum current of electroreducible ions consists only of diffusion current. The maximum current, however, consists of diffusion and additional currents. The additional current not accounted for by the formula comprises about 20% of the maximum current. The supporting electrolyte affects the diffusion coefficient of the ion to be analyzed and the higher the concentration of the electrolyte, the greater its effect.

Ya. P. Gokhshtein (1953) showed that when capillaries with a dropping period of 2-7 sec were used, the average additional current comprised 23% of the main radial diffusion current, he suggested that the Ilkovic formula for the reduction of cations and ions of the type  $H_1PbO_2^{-1}$  should be empirically modified

$$I_{\max} = 744.2 \times z \times c \times D^{1/2} \times m^{2/3} \times t^{1/6}$$

where  $I_{\max}$  - maximum current,  $z$  - Faraday number,  $D$  - diffusion coefficient,  $c$  - concentration, millimoles/1000 ml,  $m$  - mg of mercury dropping from the capillary per second,  $t$  - duration of mercury dropping.

In the case of electroreducible ions of the  $IO_3^-$  and  $BrO_3^-$  type which undergo reduction on the dropping-mercury electrode at an overvoltage, the formula is as follows:

$$I_{\max} = 465.9 \times z \times c \times D^{1/2} \times m^{2/3} \times t^{1/6}$$

Both empirical modifications may be used in cases when the foreign electrolyte contains one reducible ion only, and the rate of dropping (from the capillary) is 2-7 sec.

Owing to the diversity of the chemical composition of natural waters, it is difficult, as yet, to use the above formulas for the direct calculation of the concentration of oxygen dissolved in water. For this reason the method of calibration curves is still used. These curves are plotted from the

measurements of heights of polarographic waves in media with known concentrations of the substances to be analyzed, and under conditions of the experiment itself. Let us consider the particular case of preparing calibration curves from which the concentration of oxygen dissolved in water can be determined, 4-5 samples of water with varying concentration of oxygen, for example from 0.5 to 10 mg  $O_2$ /1000 ml, are prepared. Oxygen concentration is determined by the method of Winkler. The height of the wave and/or the diffusion current is determined for each sample. The diffusion current as a function of oxygen concentration is then plotted (Fig. 4). Thereafter the sample to be analyzed is subjected to polarographic analysis, under the same conditions as those used for the preparation of the calibration curve, and the height of the wave or the diffusion current are determined. The concentration of oxygen dissolved in water is calculated with the aid of the calibration curve (analogous curve in Fig. 15). This method is rapid and simple.

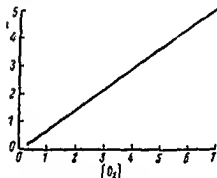


FIGURE 4 Diffusion current as a function of oxygen concentration

In 1938, Pettering and Daniels used the polarographic method for the determination of  $O_2$  in their studies on the respiration of animal tissues, blood cells, yeasts, etc., and concluded that this method was better than the manometric or Winkler's methods.

**Determination of  $O_2$  in water.** For the determination of oxygen dissolved in water a polarograph is required. Heyrovsky and Shikata (1925) described a convenient apparatus with the aid of which one can automatically register current-voltage curves on photographic paper polarograms. At present, portable polarographs with selfrecording devices are commercially available. However, not

every physiological laboratory is equipped with such an apparatus. For this reason we shall describe a simple polarograph which can be constructed in any laboratory. Such an apparatus will give results not less accurate than those obtained with automatic polarographs. The main parts of such an apparatus are: 1) a capillary tube with an inner diameter of 0.03-0.07 mm and about 30 cm long. The capillary tube should be connected with a mercury-containing bulb by means of a rubber tube, 2) electrolyzer - a chamber where the sample is analyzed, 3) calomel electrode with saturated KCl solution, 4) source of current to feed the system (accumulator at 3 volts), 5) a device for measuring the current strength (galvanometer with a scale with 200 divisions, for example Longe's galvanometer,  $10^{-8}$  ampere, inner resistance 4400 ohms, or a mirror galvanometer,  $10^{-9}$  amperes with inner resistance of about 4000 ohms, and voltage (voltmeter for 3 volts and a scale with divisions for 0.02-0.04 volts). The apparatus should be assembled as shown in Fig. 5.

Firstly, the resistance of the galvanometer shunt ought to be adjusted. For this purpose the chamber for electrolysis is filled with well-aerated water and mercury is allowed to drop from the capillary at a rate of one drop per 3-5 sec. At that time the voltage between the electrodes gradually increased

with the aid of a potentiometer (adjustable rheostat). The resistance of the galvanometer shunt should be such as to give maximum deflection of the galvanometer needle at a tension of 2 volts. The potentiometer is then returned to zero.

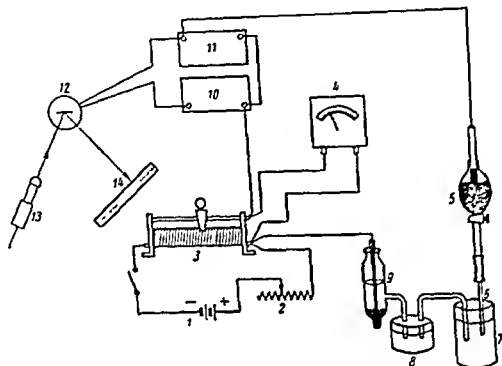


FIGURE 5. Schematic representation of polarograph

1- accumulator for 3 volts, 2- rheostat, 3- adjustable rheostat for 20-30 ohms, with easily movable contact, 4- voltmeter, 5- reservoir for mercury (separating funnel may be used), 6- capillary tube, 7- electrolytic chamber with the substance to be analyzed, 8- beaker with KCl solution and an agar bridge between the calomel electrode and the electrolyte, 9- calomel electrode with saturated KCl, 10 and 11- resistance for 10,000 ohms (one is connected in a row with the galvanometer and one parallel to the latter), 12- vertical galvanometer (sensitivity to  $10^{-9}$  amperes per 1 mm of the scale, at a distance of 1 m from the galvanometer), 13- light source for the galvanometer, 14- scale

**Obtaining of polarograms.** Having adjusted the resistance of the shunt, the voltage is gradually increased by 0.1 volt and the current strength is followed by the deflection of the needle. Having obtained the values of current strength for various voltages (to 2.3 volts for example) the potentiometer is switched off and returned to zero, and the current-voltage curve is plotted. The flattened upper part of the S-shaped curve is almost parallel to the abscissa (voltage) and expresses the magnitude of the diffusion (maximum) current.

The resistance of the galvanometer shunt remains unchanged and water with lower oxygen concentration is taken and subjected to polarographic analysis. Having analyzed 4-5 samples of water with varying oxygen concentrations (as determined by the method of Winkler) and having plotted

polarograms for each sample, the magnitude of the diffusion current characteristic for each concentration can be found. Thereafter a calibration curve is plotted. On the abscissa galvanometer readings are plotted, and on the ordinate oxygen concentration is plotted in mg or ml of  $O_2$  per 1000 ml. By joining all points, a rectilinear curve should be obtained. Thus is a calibration curve for one given temperature. Similar calibration curves may be obtained for different temperatures. In Fig. 6, calibration curves for 10, 15, 20 and 25 °C are illustrated.

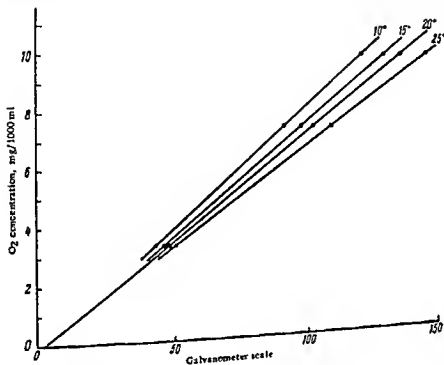


FIGURE 6 Standard curve for determining of oxygen concentration at temperatures from 10 to 25°C.

With the aid of the standard curve one can determine the concentration of oxygen in the sample. It must be stressed that the conditions under which the sample is analyzed should be exactly the same as those under which the standard solutions were analyzed.

Oxygen concentration in the sample is determined in the following way. Water to be analyzed is poured into the electrolyzer and mercury is allowed to drop from the reservoir and through the capillary. The current (from the accumulators) is switched on. Voltage is gradually increased (commencing from 1.2 to 1.8 - 2.0 volts) and the diffusion current is determined (with the aid of the galvanometer). The concentration of oxygen dissolved in the sample is determined from the calibration curve (from the corresponding diffusion current).

The form of the electrolytic chamber may vary, depending on the aim of the experiment. In the Moore and Daggar chamber (Fig. 7) mercury drops

from a capillary tube onto a mercury column placed in the outlet tube and is collected in a beaker. Through one side of the chamber platinum wire is passed, the latter is connected by means of the anode (+) to the circuit. Thus the constancy of the current in the galvanometer is secured.

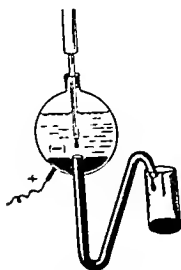


FIGURE 7 Moore and Daggan electrolytic chamber

Instead of a dropping-mercury electrode one may use rotating platinum electrodes (Kolthoff and Laitinen, 1947). The authors assumed that this electrode had certain advantages over the dropping-mercury electrode, especially for the analysis of low oxygen concentrations. Owing to the rotation of the electrode its surface regenerates constantly, and the diffusion current assumes large values and rapidly reaches a stationary state.

For studies on fish respiration, a similar electrode was employed by Stefan (1957). In her setup (Fig. 8) the rotating electrode was in the form of an elongated cone the basis of which was the working surface. The upper part of the cone was fixed to a copper wire which passed through the electrolytic chamber and was connected with a small electric motor by means of a transmission belt.

The current  $i$  on the rotating electrode depends not only on the concentration of the substance to be analyzed  $c$ , but also on the angular velocity of rotation (of the electrode)  $\omega$

$$i = A \times \omega^{1/2} \times c, \text{ where } A \text{ is constant.}$$

To ensure reproducible results, M. Stefan amalgamated the electrode with mercury before each determination and polished their surface to mirror shine with a filter paper.

In collaboration with the student I. M. Epshtein (1953), we used carbon electrodes (instead of calomel ones) for the polarographic determination of  $O_2$ . Replacement of the agar bridges with KCl (which may affect the physiological state of the experimental animal) by physiologically indifferent electrodes should prove of interest for physiological studies with animals which involve determinations of oxygen in chambers in which the animal is placed. It has been shown that in polarograms a carbon electrode gives the same current as a calomel electrode. The diffusion current is also exactly the same, but the voltage necessary to produce this current increases by approximately 0.25 volts.

The carbon electrode used by us was made from a piece of graphite 1 cm<sup>2</sup> in cross-section. Its upper end was inserted into a rubber tube containing wire from the apparatus circuit and mercury to allow contact between the wire and graphite. Before the experiments, the surface of the graphite was polished with leather.

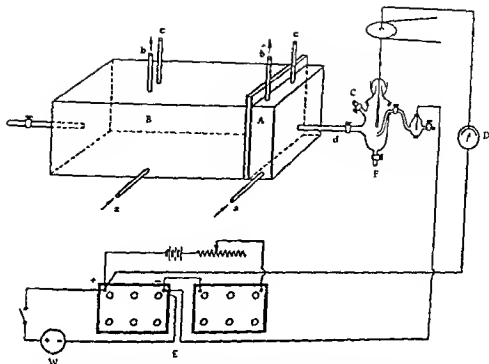


FIGURE 8. Schematic representation of Stefan's apparatus for studies on fish respiration

Chambers A (small) and B (large), e and b, tubes through which water flows in and out, C - tubes through which gaseous nitrogen is fed into the chamber during sample withdrawals through the tube d, E - measuring device for determination of the concentration of oxygen dissolved in the water of the chamber (E) D - galvanometer.

The use of an immobile platinum microelectrode (platinum wire 1 m long and 0.5 mm in diameter) instead of a dropping-mercury electrode showed that the polarogram obtained with the platinum microelectrode was shifted to the left (lower voltage) and the magnitude of the diffusion current was somewhat smaller as compared to the polarograms obtained with the dropping-mercury electrode. Diffusion current at various oxygen concentrations, as determined with the platinum microelectrodes, also gave a straight line, similar to that obtained with the dropping-mercury electrode. One and the same galvanometer reading, obtained with mercury or platinum electrodes, however, shows two different concentrations of oxygen, namely the oxygen concentration at any reading of the galvanometer obtained with platinum microelectrode was higher by 0.5 mg of oxygen per 1000 ml than that the same galvanometer reading obtained with the mercury electrode. One can use platinum microelectrodes instead of dropping-mercury electrodes, provided the calibration curve is plotted from data obtained with platinum microelectrode.

When one uses a platinum microelectrode instead of the dropping-mercury electrode and a carbon electrode instead of the calomel one, the calibration curve (diffusion current versus oxygen concentration) should also be plotted from data obtained with these electrodes. Otherwise, a large error may be introduced.

We have studied the effect of pH and water salinity on the accuracy of polarographic determination of  $O_2$ . We have determined the  $O_2$  content in waters of various salinity, and pH by means of the polarographic and Winkler methods. It was shown that the results coincided when the water salinity ranged from 0 to 30‰ and the pH from 3.5 to 13.7.

#### DETERMINATION OF ACID

Analytical methods for the determination of  $CO_2$  given off by fish are not quite satisfactory and lag behind considerably as compared with the methods proposed for the determination of  $CO_2$  in the air.

When  $CO_2$  dissolves in water it immediately combines with the latter, giving carbonic acid  $H_2CO_3$  ( $CO_2$  - pseudoacid), which then enters in chemical combination with various bases, to form carbonates and bicarbonates. This chain of transformations is under the control of the pH of the medium. The higher the pH, the lower the concentration of  $CO_2$  and  $H_2CO_3$  in water. The following graph shows the dependence of the existence of  $H_2CO_3$  in water on the pH of the medium (Fig. 9).

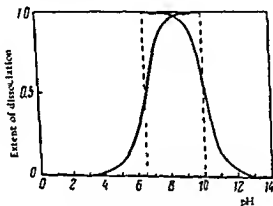


FIGURE 9 First dissociation of  $H_2CO_3$

At a pH of 4.46 and less bicarbonate is nonexistent, the only forms present being  $CO_2$  and  $H_2CO_3$ . At a pH from 4.46 to 8.37,  $CO_2$ ,  $H_2CO_3$  and  $HCO_3^-$  may be present in various proportions (the concentration of  $CO_3^{--}$  may be ignored). At a pH over 8.37, only  $HCO_3^-$  and  $CO_3^{--}$  are present (the presence of  $H_2CO_3$  may be ignored). At a pH of 8.37, 97.5% of  $CO_2$  present is in the form of bicarbonate.

We are interested in  $CO_2$ , and this, when given off by fish, is transformed in water to other forms, remaining only in small amounts in the form of  $CO_2$ .

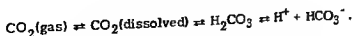
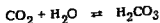
Methods for determining of  $CO_2$  are based mainly on the three following principles.

- 1) decomposition of all carbonates and bicarbonates by strong acids (for example HCl) and trapping of the  $CO_2$  liberated in barium hydroxide or sodium hydroxide. Subsequent determination is carried out by volumetric (gasometric), chemical (titration) or electrometric methods (change in electrical conductivity of  $Ba(OH)_2$  solution). The chemical and electrochemical methods are laborious and slow. They are thus unsuitable for serial determinations (in experiments on respiration).



2) The interdependence between  $\text{CO}_2$  concentration, pH and salinity (in the case of fresh water, bicarbonate concentration may be taken) is expressed by the "Henderson-Hasselbach equation", which may be derived from the following reactions

Carbon dioxide ( $\text{CO}_2$ ) dissolves in water with the formation of  $\text{H}_2\text{CO}_3$  as follows:



From the latter a dissociation constant can be calculated

$$K = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}, \quad (1)$$

where  $K$  - the first dissociation constant.

An equilibrium exists between  $\text{CO}_2$  and  $\text{H}_2\text{CO}_3$  whereas the dissociation of  $\text{H}_2\text{CO}_3$  is negligible, so that for practical purposes the concentration of remaining undissociated molecules may be assumed to be equal to the concentration of  $\text{CO}_2$ . The above equation may thus be written in the following way:

$$K_1 = \frac{[\text{H}^+][\text{HCO}_3^-]}{[\text{CO}_2]}. \quad (2)$$

If one takes  $-\log$  of both sides:

$$-\log K_1 = -\log [\text{H}^+] - \log [\text{HCO}_3^-] + \log [\text{CO}_2]$$

One may solve this equation for  $[\text{CO}_2]$  and since  $-\log [\text{H}^+]$  is termed pH and in analogy  $-\log K_1$  is termed  $\text{p}K_1$  then:

$$\log [\text{CO}_2] = \text{p}K_1 - \text{pH} + \log [\text{HCO}_3^-] \quad (3)$$

This equation combines the concentration of free carbonic acid  $[\text{CO}_2]$ , concentration of bicarbonate  $[\text{HCO}_3^-]$ , and pH. We want to calculate  $\text{CO}_2$ . To this end we have to calculate the pH of the solution, the concentration of bicarbonates, and  $\text{p}K$ .

For accurate calculations the coefficient of bicarbonate activity ( $\varphi$ ) should be accounted for. Under these conditions -

$$pK_1 + \varphi = pK^-.$$

The entire equation will assume the following form:

$$\log [CO_2] = pK^- - pH + \log [HCO_3^-]. \quad (4)$$

Concentrations of  $CO_2$  and of bicarbonate are expressed in moles per 1000 ml.

The value of  $pK$  varies (as determined by various authors). The most reliable value of  $pK$  at infinite dilution is 6.317 at  $38^\circ C$ . With the increase in concentration of bicarbonates  $pK$  somewhat decreases, this decrease being very small. When the concentration of bicarbonates does not exceed 0.1 m (the decrease amounts to 0.008) this correction may (in many cases) be ignored.

The value of  $pK$  changes with the temperature. It has been noted that when temperature becomes lower the  $pK$  will increase. The increase in  $pK$  with decreasing temperature is not strictly linear.

For practical purposes one may use the following corrections for temperature: in the range from  $38^\circ C$  to  $20^\circ C$  the increase due to temperature is 0.005 units per one degree  $C$  as the temperature is lowered, in the range from  $20^\circ C$  to  $10^\circ C$ , one has to add 0.006 to the  $pK$  of 6.317 per each degree  $C$ ; between  $10^\circ C$  and  $0^\circ C$  the correction is 0.010. In equation 4, the second dissociation constant of carbonic acid is not accounted for:  $HCO_3^- \rightleftharpoons H^+ + CO_3^{--}$ . According to Warburg, at a  $pH$  below 8 this correction is very small.

The use of equation (4) for the determination of  $CO_2$  is subject to certain limitations, since it employs a term for bicarbonate concentration which it is not always possible to determine accurately. Again in the majority of cases the error arising from this is negligible and may be ignored.

We attempted to apply this equation in the determination of  $CO_2$  given off by fish in the course of respiration. These attempts were unsuccessful, since the results obtained were unreliable. This equation enables one to determine the concentration of  $CO_2$  present in water under the given conditions, but does not allow for the accurate determination of the amount of  $CO_2$  given off by the organism and partly transformed in water into other forms.

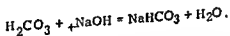
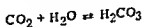
The third method for determination of  $CO_2$  is based on volumetric analysis titration of the sample with alkali in the presence of phenolphthalein. This method has its drawbacks, but can be used in certain cases. We tested this method, taking into consideration the nature of links illustrated in Fig. 23. However, to avoid low figures one must introduce a correction for the bond between  $CO_2$  and ammonia liberated ( $CO_2$  and  $NH_3$  form  $NH_4HCO_3$ ). We modified this method as follows: 150 ml pyknometer-beaker from pure white glass is filled with a dilute phosphate buffer at  $pH$  8.37, 5 drops of 1% alcohol solution of phenolphthalein are then added. The phosphate solution

assumes a weak pink color. This serves as an external indicator.

A second similar pyknometer is filled with 100 ml of water to be analyzed, and 5 drops of phenolphthalein are then added. Water is carefully poured into the pyknometer by means of a Mohr pipet and stirring is avoided. The solution is then titrated with a 0.02N solution of NaOH until it matches the color of the standard (i.e. to pH 8.37, see Fig. 9). The color should persist for at least 10 min. If the color tends to disappear, titration with NaOH should be continued.

This method, which seems simple at a first glance, calls for continued attention and careful work. To obtain reproducible results (within 0.02 ml) the sample should be mixed by gently rotating the stoppered pyknometer. The air in the room must be clean, and during titration one must avoid breathing into the titration vessel. Graduations on the buret should be easily read. A buret with 0.02 ml divisions should be protected from CO<sub>2</sub> in the air.

**Calculation.** This method is based on the following reactions



When 97.5% of carbonic acid is transformed into sodium bicarbonate, i.e. when the pH of the solution is 8.37, phenolphthalein assumes a weak strawberry-red color and titration is discontinued. Hence 1 gram-mole of NaOH combines with one gram-mole of CO<sub>2</sub>, i.e. 1N NaOH corresponds to 44 g of CO<sub>2</sub>. Hence the calculation is carried out according to the formula.

$$\frac{n \times F \times 0.88 \times 1000}{100} = n \times F \times 8.8 \text{ mg CO}_2 / 1000 \text{ ml},$$

where  $n$  = ml of 0.02N solution of NaOH used up for titration of a 100 ml sample;  $F$  = correction for 0.02N of NaOH.

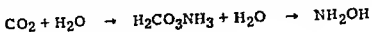
The factor 0.88 is obtained by multiplying 0.44 by 2 and corresponds to 1 ml of a strictly 0.02N solution of NaOH.

We calculate the amount of CO<sub>2</sub> per 1000 ml of water. This must be remembered, since in experiments on respiration one must measure the difference between CO<sub>2</sub> concentration prior to and after the experiment and multiply the value obtained by the volume of the vessel used for the experiment, which as a rule never exceeds 1000 ml.

Having determined CO<sub>2</sub> titrimetrically one must determine the amount of CO<sub>2</sub> given off by the organism, but combined with alkali metals.

We have carried out numerous electrometric determinations of pH in water in which fish respired and have always noticed a decrease in the pH of water. This decrease varied, depending on the ratio between the volume of the fish and the water volume, on the duration of the experiment and on the buffering properties of the water used in the experiment.

From the results of our studies, we have concluded that the ammonia liberated combines with  $\text{CO}_2$  given off according to the formula:



i. e. that 14 mg of ammonia nitrogen bind 44 mg of  $\text{CO}_2$  or in other words 1 mg of ammonia nitrogen corresponds to 3.14 mg of  $\text{CO}_2$ . Hence to obtain a true value of  $\text{CO}_2$  the amount of ammonia nitrogen (in mg) should be multiplied by 3.14 and the product added to the amount of  $\text{CO}_2$  as determined by titration. Hence the amount of  $\text{CO}_2$  given off by the fish is comprised of  $\text{CO}_2$  which can be determined by titration with NaOH and of ammonia-bound  $\text{CO}_2$

Calculation of  $\text{CO}_2$  given off by fish in mg of  $\text{CO}_2/\text{g}$  body weight/hour:

$$\frac{[n \times F \times 8.8 + 3.14 m]V}{P \times t} = \text{CO}_2, \text{ mg/l g body weight of the fish per hour}$$

where n - ml of NaOH used up for titration of a 100 ml of the sample;

F - correction for 0.02N solution of NaOH;

V - volume of water (in liters) in which the fish respired;

m - difference between ammonia nitrogen concentration (mg/1000 ml) in water prior to and after the experiment ( $m = m_1 - m_0$ ),

P - weight of the fish, in g;

t - duration of the experiment, in hours.

To express the amount of  $\text{CO}_2$  not in milligrams, but in milliliters, the value obtained should be divided by 1.964, or the following formula should be used:

$$\frac{[n \times F \times 4.48 + 1.6 m]V}{P \times t} = \text{ml of CO}_2 \text{ per 1 g of body weight of the fish per hour.}$$

The amount of  $\text{CO}_2$  given off may be calculated in grams or milliliters, according to the aim of the experiment. To determine the respiratory quotients  $\text{RQ} = \frac{\text{CO}_2}{\text{O}_2}$ , however, the amount of oxygen taken up and that of carbon dioxide given off must be calculated in milliliters.

#### PREPARATION OF WATER WITH LOW CONCENTRATION OF DISSOLVED OXYGEN

For certain experiments (for example for the determinations of threshold concentrations of oxygen in water) it is desirable to prepare water with a low oxygen concentration. This can be achieved by means of various methods. Four of these will be described. The first three methods we have used are quite simple.

1. Boiling. With increase in temperature the solubility of gases in water decreases. Thus, at  $15^\circ\text{C}$  and at atmospheric pressure water in contact with the air contains 10.3 mg of  $\text{O}_2$  per liter, and at  $100^\circ\text{C}$ , 3.4 mg of  $\text{O}_2$  per liter. This property can be used for our purposes. The setup

for preparing water with low oxygen concentration by means of this method is shown in Fig. 10.

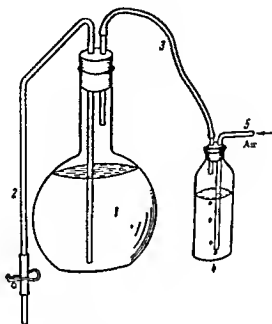


FIGURE 10 Setup for preparing water with low oxygen concentration by boiling

Water to be used in the experiments is poured into a flask and boiled for 2-4 hours. The flask (1) is then immediately plugged with a stopper provided with two tubes. Water may be withdrawn through the syphon (2), while the tube marked 3 is connected with an alkaline solution of pyrogallol (4) to free the air of oxygen when air passes into the cooled flask (1) during sample withdrawal.

This method for preparing water with low concentration of dissolved oxygen is very simple, but has two main drawbacks: 1) boiling destroys the carbonate system, as a result of which part of carbonates ( $\text{CaCO}_3$ ,  $\text{MgCO}_3$ ) becomes insoluble and is precipitated, this phenomenon cannot always be ignored, 2) the concentration of oxygen dissolved in water cannot be lowered beyond a certain point. This method is therefore unsuitable for experiments in which water with an extremely small oxygen content is required.

2. Flushing with inert gases. This method is based on the fact that oxygen dissolved in water can be displaced by blowing inert gases through the solution. Flushing with a strong stream of nitrogen is widely used. This method enables one to prepare large amounts of water with a markedly lowered oxygen concentration, while the water itself remains little changed chemically by this procedure. By means of this method the concentration of oxygen may be easily lowered to 0.2 mg in  $\text{O}_2/1000 \text{ ml}$ . If pure nitrogen is used, the oxygen concentration can be lowered to 0.1 mg in  $\text{O}_2/1000 \text{ ml}$ . When the nitrogen used is not very pure, the flushing can reduce the oxygen content to 0.5-0.7 mg in  $\text{O}_2/1000 \text{ ml}$ .

To prepare water with low oxygen concentrations, oxygen-free nitrogen from a tank is passed through water under pressure. In numerous experiments the heightened concentration of nitrogen dissolved in water can be ignored, because nitrogen is an inert gas and its narcotic effect on animals is only evident at very high concentrations. Water prepared in this way should be protected against oxygen from the air by means of pyrogallol (see method 1)

3 Passing of gaseous nitrogen through boiled water. By combining the two methods described above, i.e. when boiled water is flushed with gaseous nitrogen under pressure, the concentration of oxygen (not nitrogen) may be lowered to traces.

Cooled water should be protected against air-oxygen by the pyrogallol method described above.

Special attention must be directed to the protection of oxygen-free water from atmospheric oxygen, especially during storage. Oxygen-free water may absorb oxygen even during filling up of vessels used in the experiment, through contact with the atmosphere.

4. Addition of sodium sulfite. Water with traces of oxygen may be prepared in the following way: sodium sulfite is added to water with neutral or weakly alkaline pH. Five grams of solid salt per 1000 ml of water are used. The vessel is stoppered and mixed. When the air phase between the surface of water and stopper is small, the entire amount of oxygen dissolved in water disappears in a matter of 5 - 10 min. This method is the most efficient for preparing oxygen-free water. It is, however, not always suitable for physiological experiments, because the addition of  $\text{Na}_2\text{SO}_3$  changes the chemical composition of water (the concentration of  $\text{Na}^+$ ,  $\text{SO}_3^{--}$  and  $\text{SO}_4^{--}$  ions increases).

#### METHODS OF STUDY OF FISH RESPIRATION USING CHEMICAL METHODS OF OXYGEN DETERMINATION

Taking into consideration all the features of existence of fish, the known methods of study of fish respiration may be divided into two large groups:

1) studies on fish respiration in stagnant water and 2) in running water. Both methods have long been known and each has its advantages and disadvantages.

Choice of suitable method is of great importance. For example, manometric techniques are suitable for studies on small objects (eggs, larvae, fry etc.) but are unsuitable for respiratory studies on large fish (sturgeons, salmon, etc.). Ecological properties of the fish studied at a given stage of development should also be considered. It is true that the researcher always deals with conditions created artificially. To prevent artefacts, the researcher should endeavor to duplicate natural conditions as close as possible. The technique used (determination of  $\text{O}_2$ ,  $\text{CO}_2$ ,  $\text{NH}_3$ , mobility, respiratory rhythm etc.) is also of great importance. Accurate data on gaseous exchange cannot be obtained without an accurate analysis of the above indexes. Hence, while choosing methods for studies on fish respiration one must be guided by physiological, ecological and biological properties of the subject, and its size must also be considered.

The fish is placed in an hermetically-sealed vessel of water filled with all the above-mentioned precautions. After a given time the concentration of the dissolved oxygen is determined. If the oxygen concentration is determined before and after the experiment, the difference will be the amount taken up by the fish. This extremely simple method nevertheless calls for certain precautions.

The bacteria contained in natural waters take up oxygen dissolved in the vessel; this bacterial respiration should be accounted for, using a control vessel without fish. The water is mixed well and syphoned (at the bottom of the vessel) into 2 identical vessels, so that water overflows. The fish is placed in a full vessel (experiment no. 1) which is immediately closed to prevent air bubbles, and the time recorded. Part of the water will be displaced. The other vessel (control no. 1) is closed similarly. Both are placed in a thermostat (bath) so that temperature is constant.

At the end of incubation the experimental vessel is carefully opened, a water sample is poured into a pyknometer (a flask with a ground glass stopper) and analyzed for oxygen (see p. 27, 33), water from the control is similarly analyzed. The fish should be allowed to consume 30% of the dissolved oxygen, duration should thus be at least 2 hours. Only in special cases may shorter the duration of the experiment, the greater the error of determination. Assuming that the beginning and end of the experiment are recorded with an accuracy of 30 sec, if duration is 1 hour, error will be 1.5%, if 2 hrs, error will be 0.8%. The placing of fish and sealing of vessel, as well as withdrawal of samples should be done with maximum speed. All objects necessary should be prepared in advance and kept at hand.

Each experiment should be duplicated. The same fish is used twice in succession in different vessels. Control vessels are used each time, their oxygen concentration is the pre-experimental one. The oxygen concentration in the experimental vessel is the post-experimental one.

When control vessels are used, only the post-experimental oxygen amount is determined, however, oxygen content of both vessels must be determined. Bacterial and protozoan oxygen uptake is assumed as identical in both vessels.\* For the calculation, the difference between oxygen amount in the control vessel ( $m_0$ ) and in the experimental vessel ( $m_1$ ) is used. Oxygen amount is in mg/ml. The amount of oxygen taken up by the fish weighing ( $p$ ) during the experiment ( $t$  hours) is obtained by multiplying this difference by volume of the experimental vessel ( $V$ ). Respiratory activity is expressed in mg of oxygen per unit of weight and time.

$$\frac{(m_0 - m_1)}{p \cdot t} V = \text{MO}_2 \text{ (in mg of O}_2 \text{ per 1 g of body weight per 1 hour).}$$

For details see the chapter on O<sub>2</sub> determination in water.

\* Theoretically it can be assumed that the number of bacteria and protozoa in the experimental vessel will differ from that in the control vessel, because of fish excreta; hence this value will differ from that of the control vessel.

For experiments on respiration carried out by means of this method, vessels of various size and form may be used. All vessels, however, must be provided with ground glass stoppers. The stopper should fit the neck of the vessel so that no air bubbles will form under it. The choice of form and size of the vessel depends primarily on the properties of the fish to be studied. When small fish are studied (bleaks, small roaches, chubs etc.) vessels of any width may be used. Vessels with wide bottom are preferred for experiments with loaches, perch and other fish. In spite of the resting condition the fish are protected from light and noise; taps at the floor and walls are avoided. The first fish may at first move vertically and stir the water in the vessel, while the others usually sit motionless on the bottom of the container; concentration of  $O_2$  in the upper layers will differ from that in the lower layers of the vessel. The upper layers will be richer in oxygen than the lower ones, and the fish will respire under hypoxic conditions. This alone may change the experimental conditions. In addition, the samples for analysis are withdrawn from the lower layers of the vessel, while the oxygen content is calculated for the entire vessel. Such a gradient of oxygen concentration may lead to considerable errors. It thus seems clear that a wide and shallow vessel is preferable to a narrow and high one. The total volume of the vessel should be chosen so that the oxygen content before the experiment will exceed 3-4 times the amount of oxygen consumed by the fish in the course of 2 hours or more. To ensure uniform oxygen distribution in the experimental vessel we have proposed a modification of this method. Our modification requires that the water in the vessel be constantly and gently stirred by a stirrer rotated by means of a small electric motor. Such an apparatus is presented in Fig. 11. As can be seen, stirring of water in the experimental vessels is the only modification proposed. A protective sheet is placed between the stirrer and the bulk of the vessel.

The advantage offered by this modification lies in the fact that the concentration of oxygen in all parts of the vessel is uniform during the entire experiment. The drawback is the movement of water during the experiment. Fish living in stagnant waters (carp and crucians) react to water movement by changes in respiration rate. In experiments with fish living in stagnant waters the stirring should therefore be slow and gentle.

E. A. Veselov (1949) used the principle of running water in the respiration chamber for studies of the rate of respiration in small crucians (weighing from 1 to 12 g) in water of varying salinity. This author described a slightly modified apparatus (Fig. 12). Water in the reservoir A is well-aerated with the aid of a water pump (tube I, the direction of air stream is indicated by arrows). This reservoir is connected with the respiration chamber B through tube K. Through this tube water may pass from reservoir A into chamber B. Water from chamber B can be removed through tube L. Temperature in chamber B is maintained constant by placing the former in a water bath C. Volume of the reservoir A is 4-5 liters, volume of the chamber B - 654 ml.

To obtain mean values of respiration rate, E. A. Veselov placed 10 crucians in the chamber B. Oxygen content was determined by the method of Winkler.

In what follows, the author's description of the use of this apparatus is given: The respiration chamber B is filled to  $3/4$  of its capacity with water from reservoir A. Doing this, the clamps on tubes K and L are open. Then the stopper of the respiration chamber is opened, and a sample of water is



into a chamber for experiments with running water, by replacing the clamps on the tubes K and L by means of screw clamps. The rate of flow of water through the respiration chamber can be regulated with the aid of the clamp on the tube L. The clamp on the tube K may remain open altogether. In some experiments such a passage from respiration in stagnant water to respiration in running water may prove of certain interest.

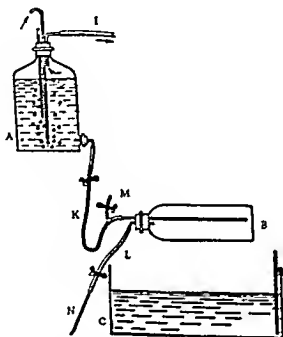


FIGURE 12. Apparatus for respiration studies (after Veselov, 1949)

A-reservoir with reserve water; B respiration chamber, C-thermostat (water bath). For other designations, see the text.

The main drawbacks of this apparatus are as follows: 1) the fish are disturbed during sampling; 2) considerable error is possible because the duration of the experiment is short (10 - 15 minutes), while the time of beginning of the experiment and its end cannot be accurately determined; 3) during repeated experiments the fish are in "old" water which is only partly replenished; 4) one cannot be sure that the test for oxygen content before the experiment gives an exact indication of the oxygen content in the water before the experiment.

#### EXPERIMENTS WITH FISH IN RUNNING WATER

In these experiments it is essential that the water runs constantly through the respiration chamber in which the fish is placed. By determining the

content of oxygen in the inflowing and outflowing water, one can calculate the amount of oxygen taken up by the fish. This will equal the difference multiplied by the rate at which water flows through the respiration chamber (ml/hour).

Krogh's apparatus (Krogh, 1916) (Fig 13) and its modification (Fig 14) has been widely used. The fish are placed in chambers through which the water is allowed to flow.

In Krogh's apparatus, the fish is placed in respiration chamber 2 and water flows in from reservoir 1. Temperature in the respiration chamber is kept constant by immersing the chamber in a water bath 4. Water having passed from reservoir 1 through respiration chamber 2 is collected in collector 3, filled with vaseline to prevent any contact between water and oxygen from the air. For quantitative analysis of oxygen content, water may be taken from tube 5 or 9. Prior to the experiment water is withdrawn through tube 5, and after the experiment through tube 9. Oxygen is determined by the method of Winkler.

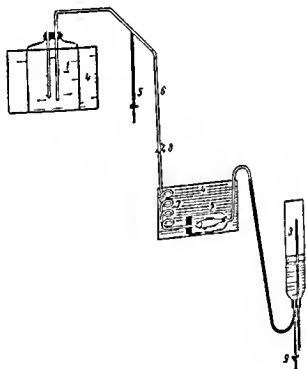


FIGURE 13 Krogh's apparatus (for studies on fish respiration) For legend, see Fig 14

The principle proved valuable, and has been used by a number of workers, but the apparatus itself has not been used. Its main drawback is that the rate of flow of water through the respiration chamber diminishes continuously.

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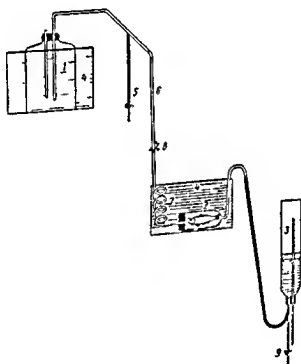


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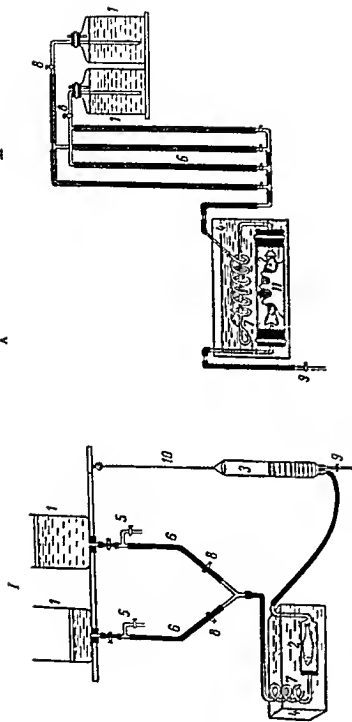


FIGURE 14a. Modified Jaeger's apparatus

i-Gaarder modification; 11-Jordan modification; 1-water bath (thermostat); 2-syphon for withdrawing water samples before the experiment; 3-collector from which water is taken for analysis; 4-water bath (thermostat); 5-syphon for withdrawing water samples before the experiment; 6-tubing through which water passes into the respiration chamber through coiled glass tubing(7); 8-screw clamp; 9-syphon for withdrawing samples from the collector (after the experiment); 10-syphon; 11-a device for fish immobilization

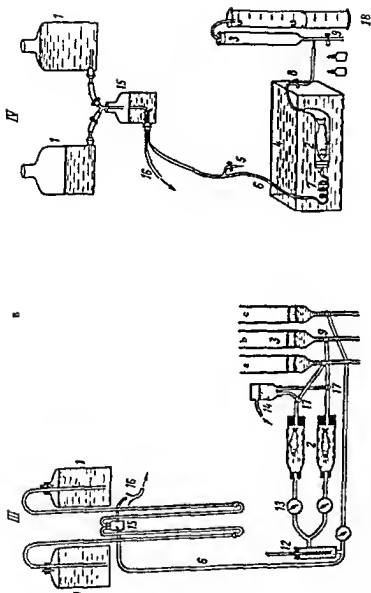


FIGURE 14b. Modified Krogh apparatus

III A Key modification; IV Sirogasov modification

1—water used in respiration experiment; 2—respiration chamber; 3—collector from which water is withdrawn for analysis; 4—water bath (thermostat); 5—syringe for withdrawing water (sample) before the experiment; 6—tubing through which water passes into the respiration chamber through coiled glass tubing (7); 8—screw clamp; 9—syringe for withdrawing water samples from the collector (sample after the experiment); 10—spring; 11—device for fish immobilization; 12—thermometer; 13—water gauge; 14—collector and outlet for water; 15—stopcock; 16—manometer (for maintaining the difference between the level in the reservoir and collector and outlet for water); 17—three-way stopcock; 18—cylinder for determining the rate of flow and amount of water passing through the respiration chamber

because the difference in water level in the reservoir and the collector tend to diminish (the level in the reservoir falls and that in the collector rises).

Attempts to eliminate this drawback were made by Gaarder (1918) who suspended the collector at a height. The best result was obtained by Keys (1930) who designed a special device to maintain a constant level in the water reservoir.

In experiments with larger fish such as sterlets, sturgeons, and stellate sturgeons, the rate of water flow through the respiration chamber must be increased considerably (to 2000 liters/hour). When the rate of flow exceeds 5000 ml/hour the collector is not necessary. Water samples for analysis can be taken directly from the tube passing from the respiration chamber. A number of respiration chambers are depicted in Fig. 15.

In 1941, we studied respiration of sexually mature sterlets under field conditions, by means of the apparatus illustrated in Fig. 16. It is simple and gives good results. All parts of the apparatus can be easily dismantled and reassembled, making it very practical for field work.

**Procedure.** This method proved more suitable for respiration studies in pelagic forms and fish living in running water than for studies on fish living in stagnant waters. The fish are prepared in the same way as for experiments on respiration in stagnant water. First, the amount of water necessary for the entire experiment is prepared. To this end water is well mixed, saturated with oxygen (water with any other concentration of oxygen may be prepared) and brought to the desired temperature. Then the apparatus is assembled (the type of chamber used depends on the size of the fish). The apparatus is filled with water, the chamber is opened and the fish placed in it. Because of the unusual conditions, the fish at first shows signs of restlessness, but the continuous inflow of fresh water calms it down and it then remains calm for the rest of the experiment. Restlessness may reappear if the supply of water is dangerously lowered and hypoxic conditions prevail, or when the fish is irritated by noise, knocks, taps, light or shadows. Noises and taps must be eliminated and the respiration chamber must be protected from excessive light. The rate of water flow must be adjusted so that the concentration of oxygen in the outflowing water will be approximately 30% lower than that in inflowing water. Usually, the inflowing water is saturated with oxygen (characteristic for each temperature). This of course does not apply to experiments aimed at studies on fish respiration under conditions of oxygen deficiency.

During its first few hours in the chamber, the respiration rate of the fish increases. When the fish has adapted to the new surroundings, its respiration rate decreases, after 3-5 hours in the respiration chamber the respiration rate of the experimental fish becomes constant and remains so for a long time. Figure 17 illustrates the respiration rate of the fish as a function of time spent in the respiration chamber.

Since water passes continuously through the respiration chamber, samples can be tested at any time. The rate of water flow must be calculated. The amount of water flowing through the chamber can be ascertained with the aid of a graduated cylinder and stop-watch. For this purpose, water flowing out of the chamber is collected in the graduated cylinder, and the amount of water flowing through the chamber per unit of time (one hour) is measured.

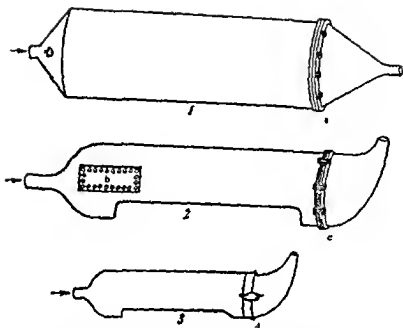


FIGURE 15 Various respiration chambers for respiration studies on sturgeons: 1-Skudovskii's chamber (for studies on large sturgeons. Chamber from galvanized iron, 2-Stroganov's chamber from sheet zinc (for studies on medium-sized stellate sturgeons); 3-Stroganov's chamber from glass (for studies on sturgeons)

a-chamber closed with screws; b-glass window, c-chamber closed with screws and nuts; d-chamber closed with ring fitted on small projections,

Respiration rate of fish during the first hours in the respiration chamber is higher than after the fish had adapted itself to new surroundings. Hence the complete curve of changes in the respiration rate of the fish in question should be obtained. This is especially important in comparative studies on the respiration rate in various fish species under similar conditions, or of one and the same fish under different experimental conditions. Stable, reproducible, characteristic and "standardized" data are necessary. Having chosen the correct rate of water flow through the respiration chamber, the experiment commences. Every hour water samples are withdrawn and analyzed for oxygen content. The rate of flow is determined 2-3 times every hour, and must be kept constant during the entire experiment unless the effect of change in the rate of flow on respiration is studied.

The rhythm of respiration movements is also determined. In the majority of fish this is done easily by counting the movements of the gill covers. During inspiration the gills are tightly covered by the gill covers, and during expiration the gill covers open to a slit through which water is expelled. Inspirations or expirations may be counted. The time elapsed is measured with a stop-watch. In this way the number of respiration movements ( $n$ ) in a certain time  $B$  (measured in seconds) is determined. For comparative studies the values  $n$  and  $B$  are calculated for one hour. If  $B$  is expressed in seconds, then the number of respiration movements in one hour ( $D$ ) will be:

$$D = \frac{n \cdot 3,600}{B}$$

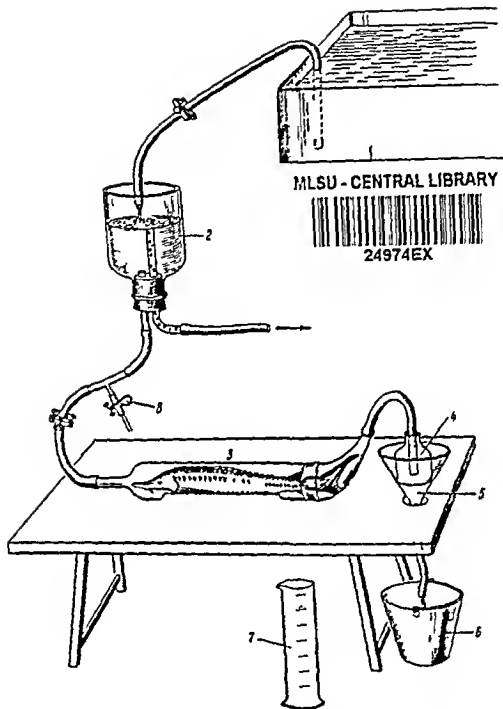


FIGURE 16 Schematic representation of an apparatus for respiration studies on sterlets. This apparatus can be used under field conditions.

1-water reservoir 2-vessel for maintaining constant water level 3-respiration chamber 4-beaker for determination of oxygen content (after the experiment) 5-funnel 6-bucket for collecting water 7-graduated cylinder 8-tube for withdrawing water samples (before the experiment)



The rhythm of respiration movements is determined every 20 minutes

At the end of the experiment the fish is taken out of the respiration chamber, it is weighed, its length is measured, and its age is determined

**Calculations** The rate of oxygen uptake by the fish is calculated according to the formula

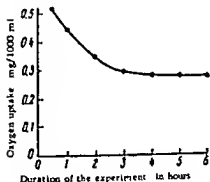


FIGURE 17 Respiration rate of fish as a function of the duration of the experiment (the time which the fish spent in respiration chamber with running water)

$$\frac{(m_0 - m_1) V}{p} = K(\text{mg of } O_2 \text{ per 1 g of body weight per one hour})$$

where  $m_0$  - oxygen content of inflowing water, expressed in mg  $O_2$ /1 ml (oxygen content before the experiment,

$m$  - the same in outflowing water (oxygen content after the experiment,

$V$  - volume of water (in ml) which passed through the respiration chamber in one hour,

$p$  - weight of the fish

If the rate of oxygen uptake is to be expressed not in milligrams but in milliliters, then both  $m_0$  and  $m_1$  must be expressed in milliliters. The other values remain unchanged

To calculate the amount of  $CO_2$  given off (or that of any nitrogenous compounds released) the following formula may be used

$$\frac{(m_1 - m_0) V}{p} = N(\text{mg of nitrogen per 1 g of body weight per one hour}),$$

where  $m_0$  - the amount of bound nitrogen ( $NH_3$ ) in the inflowing water (in mg of  $NH_3$  per 1 ml of water - before the experiment),

$m_1$  - the same in the outflowing water (the amount of  $NH_3$  after the experiment),

$V$  and  $p$  - the same as in the previous formula

For calculating the amount of  $CO_2$  given off by the fish, the same formula may be used. In that case  $m_0$  and  $m_1$  show the amount of  $CO_2$  per 1 ml of inflowing or outflowing water, respectively. The content of  $CO_2$  is usually expressed in milliliters

If the amount of  $CO_2$  given off by the fish and the amount of  $O_2$  taken up by the latter is known, the respiratory quotient (RQ) can be calculated

$$RQ = \frac{CO_2}{O_2}$$

where  $CO_2$  - ml of  $CO_2$  given off per 1 g of body weight in one hour,

$O_2$  - ml of oxygen taken up per 1 g body weight in one hour.

During "pure" carbohydrate metabolism the  $RQ = 1$ , fat metabolism yields  $RQ$  values of 0.7 and protein metabolism  $RQ$  values of 0.81. The respiratory quotient may exceed 1 or be lower than 0.7 when the physiological state of the fish is disturbed by internal or external factors (pathological states) or during changes in the physiological state of the fish (fattening, oxygen starvation etc.)

Oxygen uptake per respiratory movement (A) may be calculated in the following way

$$A = \frac{m_0 - m_1}{R}$$

where  $m_0$  - initial oxygen content (in water),

$m_1$  - final oxygen content (in water),

R - number of respiration movements of the fish during the experiment.

For comparative studies on the respiratory activity of gills in fish of varying ages, the oxygen uptake per respiratory movement (O) per unit of weight is the most representative. For this purpose the following formula may be used

$$\frac{RR}{RM} = O$$

where RR - rate of respiration,

RM - the number of respiratory movements

In experiments on respiration in running water, several respiration chambers may be placed side by side and oxygen consumption may be measured in several fish at the same time

Fry and Hart (1948, 1948a) described a very simple apparatus (Fig. 18) for studies on the rate of respiration in very small fish (goldfish): this apparatus has been used successfully by some other workers as well.

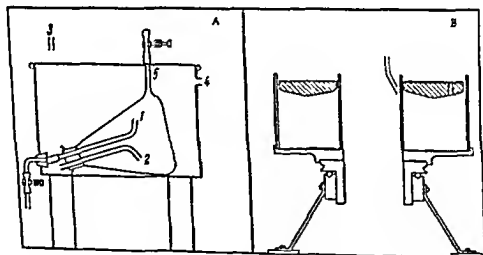


FIGURE 18 Apparatuses for studies on respiration in fish in motion constructed by Spoor (1946) and Fry and Hart (1948)

A apparatus for the determination of basal metabolic rate, B apparatus for determination of maximal metabolic rate

Apparatus A depicted in Fig. 11\* can be used for studies of basal metabolism in fish (basal metabolism during 24 hours) and on their maximum metabolism. A 2000 ml Erlenmeyer flask serves as respiration chamber. The

\* [An error in the Russian original should read Figure 18]

flask is sealed with a stopper through which two tubes (1 and 2) pass. Tube 1 is connected with another tube which passes through the lower part of the wall of the wide water bath. The water bath serves both as a thermostat and as a water reservoir for the respiration chamber. Water excess passes through a tube inserted at the upper part of the water bath wall. The fish to be studied are placed in the flask, which is immediately closed with a stopper provided with two tubes, 1 and 2. The flask is then placed on its side on the bottom of the wide water bath. Tube 1 is then connected with the tube which passes through the water bath wall (see Fig 18, A). Tube 2 is connected with the water bath through this tube water passes from the water bath and out into the respiration chamber.

Water saturated with oxygen from the air and adjusted to the desired temperature is poured into the water bath through tube 3, while the excess water is removed through tube 4. In this way a constant water level is maintained in the water bath.

If the clamp on tube 1 is opened, water from the water bath will pass through tube 2 into the respiration flask. Tube 5 is used for collecting gas bubbles formed in the flask. With the aid of the clamp on tube 1 the flow of water through the respiration chamber can be regulated.

When the experiments are conducted at 20°C or less, 20 goldfish may be placed in the chamber at one time (average weight of fish 3.8 g). At temperatures above 20°C - 10 goldfish may be taken for one experiment.

Water from the water bath (before the experiment) and from tube 1 (after the experiment) is analyzed for determination of the initial oxygen content in the water. Oxygen is determined by the method of Winkler. If the rate of water flow and the difference in oxygen content in the water in the thermostat and respiration chamber are known, the amount of oxygen taken up by the fish can be easily calculated.

This apparatus is simple to use, but has certain drawbacks. Firstly, the inlet and outlet tubes 1 and 2 are placed too close to each other, as a result, the water in the chamber is not satisfactorily mixed and one cannot be sure that the oxygen in the water taken from tube 1 represents the true oxygen content of the water "after the experiment". Secondly, fish which swim in the respiration chamber are hampered in their movements by tubes 1 and 2.

Maximal metabolism is determined in a rotating vessel (Fig 18, B), in which the fish swim against the water current. After a given time a water sample is withdrawn by means of a 100 ml pipet for analyzing the oxygen content. The sample is poured into a 50 ml beaker (25 ml are poured into the beaker and 25 ml remain in the pipet).

In studies on respiration of fish in running water the following difficulty is encountered when the rate of flow of water through the chamber is increased. The higher the rate of water flow through the respiration chamber, the smaller the difference between oxygen concentration in the inflowing and outflowing water. The smaller this difference is, the larger the error of the experiment. Hence the rate of flow should be selected so that the difference in oxygen concentration in the inflowing (prior to experiment) and outflowing (after the experiment) water will be enough to produce reliable results. In studies on rheotactic fish such a limitation in the rate of flow is undesirable.

N. S. Stroganov (1949) described an apparatus which allowed for any rate of flow (Fig. 19). Water in which the fish respire circulates within a closed system. Centrifugal pump 2 operated by an electric motor 1 feeds water

into the respiration chamber 3 and from there into reservoir 5 and back into the pump. Reservoir 5 limits the duration of the experiment, since its oxygen content is restricted. To obviate this difficulty another reservoir (duplicate 5) is added to the system, this reservoir is connected with the system through a large double three-way stopcock (Fig. 20). By using alternately one or another reservoir (5 and its duplicate), the duration of the experiment can be prolonged indefinitely without having to take the fish out of the respiration chamber.

To enable one to withdraw water samples at any time without disturbing the rate of flow and the closed circuit, reservoir 5 is provided with a rubber bladder of a football 6, the socket of which is tightly fitted on a metallic or glass tube, the free end of this tube passes through a rubber stopper in reservoir 5 and outside into the atmosphere. Reservoir 5 contains a syphon 7 through which water samples are withdrawn for analysis. When the clamp on syphon 7 is open, water flows from reservoir 5 through syphon 7 and is replaced by atmospheric air to which passes into the rubber ball, inflating it. This device allows for withdrawal of water samples for analysis without changing the total volume of the closed system. Constant temperature is maintained during the experiment by placing the respiration chamber into water bath 8.

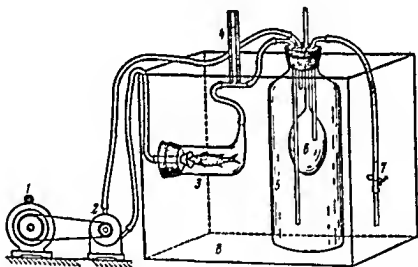


FIGURE 19 Apparatus for studies on respiration of fish placed in a closed circulation system (Stroganov, 1949)

Outside the water bath there is an electric motor (100 volts), a centrifugal pump (5–7 cm in diameter) and rubber tubes which connect the pump with chamber 3 and reservoir 5. The rate of flow of water through the respiration chamber may be regulated by means of driving pulleys of various diameters placed on the axis of the motor and the pump.

The rate of water flow in the system must be determined in preliminary experiments. For this purpose the rubber tube passing from reservoir 5 is freed, and one end connected with reservoir 5 is left on the water level in the water bath 8, while the other end is put into water in water bath 8. The rate

of flow of water at any ratio between the diameters of the pulleys of the motor and of the pump can be determined by measuring the outflowing water from reservoir 5 in a graduated cylinder.

If the amount of outflowing water (per time unit), the diameter of the respiration chamber, and the cross-section of the fish are known, the rate of flow of water through the chamber (in ml) and the linear velocity of water current in various parts of the free space between the fish and the chamber wall can be calculated.

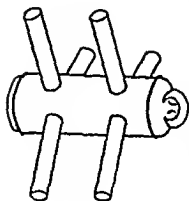


FIGURE 20. Double three-way stopcock

By introducing Pito 4 tube into the water stream, the rise in water level in the Pito tube during any rate of flow can be measured. In this way the tube is calibrated. During the experiment, calibrated Pito tubes indicate the velocity of water in a closed system at any given time. The tube through which water passes from the respiration chamber should reach almost to the bottom of reservoir 5, and the tube which passes from the reservoir to the pump should be placed in the upper layers of the water in reservoir 5. This order can be reversed. This is done to enable the water in reservoir 5 to be well

mixed, so that the sample should be representative of the water in the reservoir. Proceeding from these considerations, the end of the glass tube of syphon 7 should be lowered to the middle of reservoir 5. The volume of water in the entire closed system must be determined beforehand.

Respiration is measured in the following order. The entire system is filled with well-aerated water. The respiration chamber 3 is opened, the fish is placed in the chamber, and the chamber is closed. Chamber 3 is placed in water bath 8 and the motor is switched on. The stopper of reservoir 5 is opened, and water is poured in so that no air bubbles will be present in the entire closed system. Water samples are then withdrawn for analysis, this marks the beginning of the experiment. After a given time another sample is withdrawn for analysis. The volume of all samples must be known, in other words the entire amount of water taken from reservoir 5 must be accounted for. This amount is equal to the volume of water taken into the pyknometers, plus the volume needed for rinsing the beakers and the outflowing excess water. The volume of the pyknometer is known, water which overflows and water taken for rinsing is measured in a cylinder. Having determined the oxygen content in a pyknometer, the former is calculated for the entire volume of the closed system. The rate of oxygen uptake by the fish is calculated as described above (p. 61). In the subsequent determinations of respiration the amount of oxygen in the whole system after the experiment is accepted as the amount of oxygen before the experiment for the following determination. Since respiration takes place in a closed system, the concentration of oxygen gradually decreases. The linear decrease is noted in cases when the fish is in a resting state and the rate of flow remains constant. Respiration rate as a function of oxygen concentration is depicted graphically in Fig. 21.

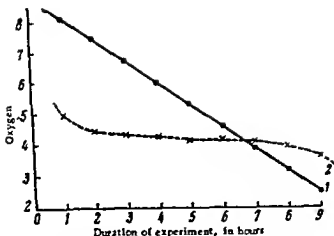


FIGURE 21 Rate of respiration in water with decreasing oxygen content

1-the content of oxygen in water in mg O<sub>2</sub>/1000 ml of water,  
2 oxygen uptake by fish (mg O<sub>2</sub>/10 g body weight/hour)

As can be seen from Fig. 21, the fish rapidly calms down and for some time the respiration rate remains constant, until the oxygen concentration reaches a certain low value, after which respiration rate decreases.

If the aim is not to study respiration during oxygen starvation, the experiment may be discontinued after a steady respiration rate had been attained. This constant respiration rate may be considered as characteristic for the fish in question under the specified conditions.

This method enables us to determine the respiration rate during various speeds of water flow through the respiration chamber, and to carry out analyses at any time intervals. The accuracy with which the total amount of oxygen is determined does not depend on the rate of water flow. These are the positive aspects of this method. Its drawback lies in the fact that respiration takes place in a constant and gradually diminishing oxygen concentration, as in experiments on respiration in "stagnant water". Hence to obtain reproducible data the experiment must be discontinued after a steady respiration rate has been attained,

#### METHODS OF STUDY OF FISH RESPIRATION BY MEANS OF PHYSICAL METHODS OF OXYGEN DETERMINATION

Completely different apparatuses are used for studies of the rate of respiration (oxygen uptake) of developing eggs, larvae, fry and small fish (the size of guppies). In some cases the experiments can be conducted in small flasks, as described on p. 51.

In laboratory practice, manometric methods are widely used. The principle of all manometric methods lies in the fact that in a closed system at a constant temperature and constant gas volume any changes in the amount of gas (gas uptake or release) can be measured by changes in its pressure. In studies on respiration of small aquatic animals we mainly limit ourselves to

experiments on oxygen uptake (decrease in gas pressure in the closed system). In Soviet laboratories constant volume Warburg or Drastich modified respirometers are mostly used.

#### THE WARBURG CONSTANT VOLUME RESPIROMETER

I. M. Sechenov's manometric method for the determination of gases in blood (the blood-gas manometer) had been improved and developed, and the widely used Barcroft and especially Warburg apparatuses have been derived from Sechenov's blood-gas manometer. The form of the Warburg apparatus is very convenient. Respirometers of a similar type are widely used in different laboratories the world over, and differ considerably from that described by Warburg. The name Warburg respirometer has been preserved, although only Warburg's principle has been maintained.

The respirometer is based on the following principle: The organism to be studied is placed in a flask to which a manometer is attached. In the course of respiration, conditions in the flask change, the oxygen content decreases and  $\text{CO}_2$  content increases (release of  $\text{NH}_3$  is ignored). If the vessel is provided with a center well with KOH solution to absorb  $\text{CO}_2$ , then the only change in gas pressure will be due to a decrease in oxygen content ( $\text{CO}_2$  will be absorbed by KOH). The oxygen concentration will decrease, and its pressure will thus decrease as well. This decrease in pressure can be easily measured with the aid of the manometer. If the change in manometer reading and the flask constant are known, the amount of oxygen taken up by the organism under study can be easily ascertained.

The Warburg respirometer (Fig. 22) consists of three main parts: 1) flask attached to the manometer (seen in the Fig.); 2) water bath at constant temperature, and 3) a shaking device to shake the flask and the manometer, to promote a rapid gas exchange between the fluid and the gas phase in the flask. The most important part are the flask and manometer. The flask is immersed in a water bath (electrically heated) together with a stirrer and two thermometers; one, a Beckman thermometer allowing for readings to  $0.002^\circ\text{C}$ , and another with readings to  $0.1 \sim 0.2^\circ\text{C}$ . The electric heater is connected with a thermoregulator (toluene thermoregulator or contact thermometer) and a relay. Current is provided by the general electric supply. The electric stirrer which works continuously is also fed from the same electric system.

The stirrer serves for stirring water in the water bath, thus ensuring a uniform temperature in all water layers. In addition there is a special device for shaking the vessels and manometers, which promotes a rapid equilibrium between oxygen tension in the aqueous phase of the vessel and its gaseous phase. Rapid gas exchange between the fluid and gaseous phase is indispensable for accurate determination of oxygen uptake by means of changes in gas pressure in the vessel.

The shaking device may differ, depending on the form of the water bath. With quadrangular baths (the most widely used) the vessel together with the manometer is shaken backwards and forwards (amplitude of 2-3 cm). The rate of shaking is approximately 100 oscillations per minute. In a circular water bath (manometers are arranged around the bath) a shaking device of rotating type is used.

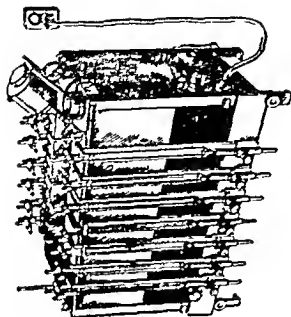
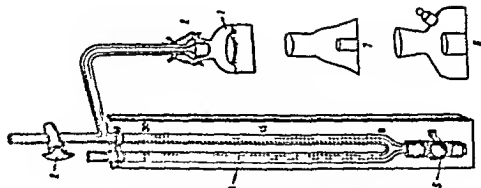


FIGURE 22 Warbu constant volume respirometer to the right - assembled respirometer to the left - manometer with flask (respiratory camera)

1 - flask; 2 - syring keeping flask attached to manometer  
3 - stopcock 4 - manometer graduated in 500 mm 5 - clamp  
on rubber tube 6 and 7 - various types of flasks





Before the experiments the working conditions of the respirometer must be ascertained. The water bath must be filled with water, the heating and the stirrer must be turned on and the contact thermometer set at the temperature desired. The toluene thermoregulator is set after the desired temperature is reached.

Now the vessels and manometers may be prepared. They should be well cleaned and dried with alcohol. The manometers are then filled with Brodie's solution (the entire lower part enclosed in rubber). The ground glass joints of the flasks and manometers are greased. Brodie's solution is a monometric fluid with a density of 1.033 compared to 13.60 for Hg (a column of this fluid 10,000 mm high equals 1 atm =  $P_0$ ). Brodie's solution is prepared as follows: 23 g of sodium chloride and 5 g of sodium choleate dissolved in 500 ml of water. A little acid fuchsin is added (to make the fluid visible).

Grease for attachment joints and plugs is made from anhydrous lanolin or thick vaseline mixed with a small amount of rubber glue.

Having adjusted the thermoregulator and after bringing the water in the water bath to the desired temperature and preparing the flasks and manometers, the gas volume of the flasks and the space in the manometers to the 150 mark are determined. In all studies on respiration rate, the flask constant must be known. For this purpose the total gas volume of the flask and the manometer to the mark 150 ( $V_0$ ) must be determined. The volume of this space is determined by filling it with Hg and weighing. By dividing the weight of mercury by its specific gravity, the volume  $V_0$  can be found. This method of flask calibration is not very convenient, however, and another method may be used.

The flask is rinsed with a small amount of distilled water. This is done to saturate the air in the flask with water vapor. The vessel is then attached to the manometer. The ground glass joints are greased. The attachment spaces (after attaching the flask to the manometer) must be transparent. The stopcock must be opened, otherwise Brodie's solution will overflow. The manometer with the attached flask is then placed in the thermostat. The manometer is still open to the atmosphere and Brodie's solution is lowered to the mark 0 (this is done with the aid of the clamp 5 on the rubber tube on the manometer Fig 22). Thereafter the flasks are shaken until the temperature of the flask is the same as that of the water in the water bath. Shaking is discontinued after 10 - 15 minutes. The menisci in the manometer are set at zero, then the stopcock is closed and Brodie's solution is brought to the mark 150 in the right arm of the manometer by squeezing the clamp on the rubber tube. In the left arm (open arm) the liquid will be somewhat higher than in the right arm. The difference is recorded. Let us assume that it is equal to  $h_1$  mm. The stopcock is again opened, the liquid in the manometer is lowered to zero, and the manometer is again shaken. After 5 - 7 minutes the entire procedure is repeated. Similar values for  $h_1$  should be obtained in 3 - 5 determinations. The stopcock of the manometer is then opened, the manometer is taken out of the water bath and the flask is disconnected. Now the flask is filled with an exact volume of distilled water (added with a volumetric pipet) which must amount approximately to one half of the total gas volume of the flask (let us assume 'a' ml). The flask is again attached to the manometer and placed in the water bath. The levels in arms of the manometers are again set at zero and the manometers are shaken. After 10 - 15 minutes, the entire procedure is repeated. The levels are set at zero, the

stopcock is closed and the liquid in the manometer is raised to the mark 150 in the right arm of the manometer. In the left (open) arm the level is higher. The difference  $h_2$  will be larger than  $h_1$  obtained with the empty flask. In this case also, 3-5 determinations should be made. As seen,  $h_1$  and  $h_2$  are determined in the same way. The only difference is that  $h_1$  was determined with an empty flask, while  $h_2$  was determined with a flask filled with 'a' ml of water. In other words, the gas volume of the flask decreased by a ml, and the gas compressed occupies less space, hence  $h_2$  is greater than  $h_1$ .

Calculation of  $V_0$  from the data obtained is based on the following theoretical considerations. According to Boyle-Marriott's law, the product of gas pressure (P) and the volume it occupies (V) is constant (k).

$$PV = k.$$

Pressure is inversely proportional to the gas volume at a constant temperature.

Gas pressure in our flask will be designated as P (the water vapor pressure is accounted for), gas volume as  $V_1$  (when the level in the manometer is at zero). Hence  $V_1P = k$  at a given temperature.

If this gas volume at constant temperature is compressed to volume  $V_0$  (to 150 mm in the right arm of the manometer before the meniscus is seen), volume  $V_1$  will decrease to  $V_0$  and pressure P will increase by  $h_1$ . The product of the new pressure and the new volume will be equal to the first product, i.e. :

$$V_1P = V_0(P + h_1).$$

If now the flask is filled with 'a' ml of water, its gas volume will decrease by a ml. By compressing the gas space, as in the preceding case, we shall obtain a new change in pressure ( $h_2$ ). Now the equation will have the following form:

$$(V_1 - a)P = (V_0 - a)(P + h_2)$$

or

$$V_1P - aP = (V_0 - a)(P + h_2)$$

As can be seen from the equation,  $V_1P = V_0(P + h_1)$ . Substituting this value in the latter equation we shall obtain:

$$V_0(P + h_1) - aP = (V_0 - a)(P + h_2).$$

By opening the brackets and after reduction, we shall obtain:

$$V_0h_1 = V_0h_2 - ah_2.$$

Solving this equation for  $V_0$  we shall obtain:

$$V_0 = \frac{ah_2}{h_2 - h_1}.$$

$V_0$  is the gas volume in the flask plus the space of the manometer to the 150 mm mark.

If at a constant volume gas is taken up or released its pressure P also changes.

$$\frac{V_0 \pm V}{V_0} = \frac{P_0 \pm P}{P_0}$$

For V it could be registered as:

$$\pm V = \frac{V_0 P}{P_0}$$

In our case the  $\text{CO}_2$  given off is absorbed by alkali, hence the volume of gas will decrease only as a result of oxygen uptake by the animals studied.

In the latter equation the ratio  $\frac{V_0}{P_0}$  is the flask constant. Flask constants must be determined for each volume of the reaction mixture in the flask (water with the object studied and the amount of KOH in the center well). Under these conditions the amount of gas taken up (in our case of  $\text{O}_2$ ) will be:

$$V = \frac{V_0 h}{P_0}, \quad \frac{V_0}{P_0} = k,$$

then  $V = hk$ .

To determine oxygen uptake by the organism under study, it is first necessary to calculate the value of  $k$  and then to determine  $h$  experimentally.

If the value of  $V_0$  is known, the flask constant  $k$  can be calculated. Prior to determination of flask constant, one must decide what final volume of liquids (water for respiration plus KOH in the center well) the flask will contain. If  $V_0$  is approximately 25 ml, the maximum volume of liquid which can be added to the flask should not exceed 10 ml. The amount of water (liquid) that must be added to the flask should be approximately determined, the peculiarities of the organisms studied and the duration of the experiment should also be accounted for. It is desirable that at the end of the experiment the amount of oxygen dissolved in water will amount to 50 - 70% of that initially present, 0.5 - 1.0 ml of alkali are added.

The calculation is carried out according to the formula,

$$k = \frac{V_g \times \frac{273}{T} + V_n \times \alpha}{P_0}$$

where  $V_g$  - volume of gas phase in flask, including connecting tubes down to the reference point - 150 mm in the closed arm of manometer, minus the volume of fluid in the flask ( $V_{fl}$ ) (reaction mixture plus alkali);

$V_{fl}$  - volume of fluid in the flask (reaction mixture + alkali),

$T$  - temperature of bath in absolute degrees ( $T = 273 + t^\circ$ );

$\alpha$  - solubility of gas in reaction liquid - in this case of oxygen in water at a pressure of one atm. - (ml of  $\text{O}_2$  in 1 ml liquid), this solubility coefficient depends on temperature: at  $10^\circ\text{C} = 0.03793$ , at  $15^\circ\text{C} = 0.03441$ ; at  $20^\circ\text{C} = 0.03031$ , at  $25^\circ\text{C} = 0.02822$ , at  $30^\circ\text{C} = 0.02612$  and at  $40^\circ\text{C} = 0.02309$ . For intermediate temperature,  $\alpha$  is found by interpolation. With increasing salinity somewhat decreases.

$P_0$  - standard pressure expressed in mm of manometric liquid.

Brodie's solution has a specific gravity of 1.033, so that

$$P_0 = \frac{760 \times 13.6}{1.033} = 10,000.$$

It must be remembered that in this formula  $P_0$  is expressed in millimeters and  $V_{\text{gas}}$  and  $V_{\text{fluid}}$  in microliters ( $\mu\text{l}$ ).

The flask constant holds only for a given set of conditions. If the temperature of the experiment or the volume of liquid added to the flask is changed, the constant will also change (this is clearly seen from the formula). In practice the following procedure is used. If  $V_0$  is known (it remains constant for long) one calculates flask constants for several volumes of liquid in the flask and for several temperatures. For the experiments proper, the corresponding constant should be used.

Having finished all preparatory work, one may begin experiments on oxygen uptake by the experimental animal.

A clean and dry flask is filled with water (the volume depends on the aim of the experiment) and the experimental animal or animals are placed inside the flask. The center well is then filled with 0.5 – 1.0 ml of alkali for the absorption of  $\text{CO}_2$ . Alkali should be added carefully so as not to spill into the main compartment where the animal is placed. Afterwards the flask is attached to the manometer (the stopcock should be open to the atmosphere). The manometer with the flask is placed in the water bath and shaken. After 10 minutes of equilibration (when the temperature inside the flask will be similar to that of the water bath) the fluid in the manometer is set at 150 mm and the stopcock closed. The manometer is shaken for the desired time. At the end of the experiment shaking is discontinued, the manometers are placed vertically, and the liquid in the right arm of the manometer (closed arm) is brought to the mark 150 by means of the clamp 5. The fluid in the open left arm will be lower than that in the right arm. The difference between the level in the closed and open arms  $h$  shows the change in the pressure of gas in the flask due to oxygen uptake by the experimental animal.

The amount of oxygen taken up ( $A$ ) is equal to  $A = kh$ . This is expressed in microliters.

Having recorded the reading of the manometer, one may continue the experiment if necessary. To do this one may open the stopcock, bring the liquid in the manometer to the 150 mark, close the stopcock and continue to shake the manometers. In this case it is as though one repeats the experiment from the first stage. One may also continue the experiment without opening the stopcock. In this case, after having recorded the reading of the manometer, the manometers are shaken, and the experiment is continued without having opened the stopcocks. In both cases it must be remembered that for the subsequent reading of  $h$  one must account for the meniscus level in the left, open arm during the first reading.

If the duration of the experiment ( $t$  hours) and the weight of the animal ( $p$  grams) are known, one can calculate the amount of oxygen taken up per one g of body weight of the animal in one hour ( $B$ ).

$$\frac{A}{pt} = \frac{kh}{pt} = B \text{ (in microliters of } \text{O}_2/\text{lg body weight/hour).}$$

The animals should be weighed at the end of the experiment only. As can be seen, the amount of oxygen taken up by the organism studied is equal to the flask constant multiplied by the manometer reading  $h$ . The accuracy of reading is 0.5 mm (equal to the flask constant – if it is assumed that the errors in reading are additive). Hence it can be concluded that the smaller the flask

constant the smaller the absolute amount of  $O_2$  which can be determined. The flask constant changes with the size of the flask. The larger the flask the larger the flask constant. The flask constant is dependent in the main on the total gas volume of the flask. Good results are therefore obtained when small flasks (15 - 30 ml) are used. Flasks 150 ml in volume and larger are unsuitable.

Temperature is another factor which strongly affects the results of the determinations. It is known that a  $1^\circ C$  change in temperature will result in a change of pressure on the gas volume by  $\frac{1}{273}$  of its initial value. It follows that if the gas volume of the flask (above the reaction mixture) is 20 ml, and the temperature changes by  $0.1^\circ C$ , the gas volume (or its pressure) will change by  $\frac{1}{2730}$  i.e. by 7.3 microliters. Hence the temperature must not fluctuate by more than  $0.002^\circ C$  in the course of the experiment.

However, such a temperature regulation is laborious and slow. To eliminate the effect of temperature changes during the experiment (as well as changes in atmospheric pressure) thermobarometers are used. Such a thermobarometer is an ordinary Warburg flask attached to a manometer. The flask is filled with water to approximately  $\frac{1}{4}$  of its volume (the volume of water is not of great importance). In other words, in all experiments on oxygen uptake an additional flask without any experimental animals inside it should be used (without reaction mixture). Changes in room pressure and temperature of the water bath are thus corrected for by the thermobarometer.

The use of a thermobarometer. The animals are placed in the experimental flask which is then attached to a manometer (the stopcock is open) and placed in a water bath for equilibration. The meniscus is set at the 150 mark of the manometer. At the same time the flask without the reaction mixture, the thermobarometer, is subjected to the same procedure. After equilibration of temperature (after shaking in the water bath, as described above) the meniscus in the experimental and control (thermobarometer) flasks are set at the 150 mark and the stopcocks are closed (beginning of experiment). At the end of the experiment (after time  $t = 15, 30$  and  $60$  minutes) the meniscus in the right arm of the manometer to which the experimental flask is attached is higher than the initial level and in the left arm lower than the initial level (due to oxygen uptake by the animal studied) (150 mm in the right arm). The meniscus in the right arm is brought down to the 150 mark and then the meniscus in the left (open arm) reads, let us say, 115. The difference is 35 mm. The thermobarometer (after the meniscus in the right arm is brought down to the 150 mark reads 147 (in the left arm) - the difference being 3 mm. This difference was brought about either by lowering of the temperature in the water bath, or else by the increased atmospheric pressure in the room or by both factors together. Since the thermobarometer has all the time been under the same conditions as the experimental flask (and the readings were taken simultaneously) it could be assumed that similar change in pressure also occurred in the experimental flask i.e. the drop in pressure in the experimental flask (in our case 35 mm) was due to oxygen uptake ( $x$  mm) and to external conditions (3 mm). Hence  $x = 35 - 3 = 32$ . As a result of respiration the amount of oxygen in the flask decreased, hence the pressure dropped by 32 mm. Multiplying this value by the flask constant, we shall obtain the amount of oxygen taken up by the experimental animal during the experiment.

If the level of liquid in the left arm of the thermobarometer had risen (due to rise in temperature of the water bath or to a drop in the atmospheric pressure) the reading of the thermobarometer is added to the value of  $h$  recorded in the experimental flask. The value obtained is then multiplied by the flask constant. In this way the amount of oxygen (in microliters) taken up by the animal studied is ascertained.

In the Warburg apparatus there is usually room for 14 flasks with manometers. This enables one to carry out simultaneous experiments with several organisms. In this case, recording of the results must be very careful. Each worker has his own method for recording results. As an example we shall present a form of recording results (during work with the Warburg apparatus), which may be useful for the beginner:

Determination of oxygen uptake in Warburg apparatus

Date .....  
 Temp. of the experiment .....  
 Flask, No. ....  
 Animal .....  
 Experimental conditions (fluid in which animal is kept water, saline etc) ...  
 Number of animals .....  
 Flask volume .....  
 Volume of KOH .....  
 Weight .....  
 Water volume .....  
 Flask constant .....

Time of reading	Thermo-barometer	Flask 1						Flask 2						Flask 3					
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6

The figures in the columns "flask 1", "flask 2" and "flask 3" show: 1-reading, mm; 2-change in the pressure of the flask,  $h$ ; 3-correction for thermobarometer, 4-change in pressure of the flask due to oxygen uptake; 5-oxygen taken up, 6-oxygen uptake in microliters/g body weight/hour.

Respiration rate may be measured by the total uptake method and the interval uptake method. The total uptake is calculated by subtracting the initial reading from all subsequent readings. The rate is calculated from the beginning of the experiment. The interval uptake method is based on the procedure of subtracting each reading from the one following it. The value obtained and corrected for the flask constant is calculated per hour and 1 g body weight of the fish studied. By using this method, the kinetics of respiration in the course of the experiment may be studied. This cannot be studied by the total uptake method, since for this method additional calculations are needed.

In experiments carried out according to the first variant, the manometer stopcock should be closed during the duration of the entire experiment, from the beginning to the end. With the second variant the stopcock may be kept closed as in the first variant, or at each time interval the stopcock may be opened and the meniscus set at the 150 mark. then the stopcock is closed, and the shaking continued.

Draatich modified Barcroft's blood-gas manometer so that it can be used as a microrespirometer (Fig. 23).

The method is based on the following principle: The apparatus contains two identical flasks A and B. One flask contains the experimental animal. The flasks are connected with each other by means of a tube (1) inside of which there is a drop of liquid (colored paraffin) (2) or, as it is called, an "index". If  $\text{CO}_2$  given off by the experimental animal in flask A is absorbed by KOH solution placed in a center well (or by filter paper soaked with KOH), the pressure of the air above the liquid will decrease owing to oxygen consumption by the experimental animal. As a result, a difference between the pressure in flask A and flask B will be noted (in flask B the pressure remains unchanged). This difference in pressure will tend to level off and the drop of liquid (2) in the tube connecting both flasks will move from B towards A. If the diameter of the connecting tube and the distance which the liquid moves are known, the volume by which the pressure in the experimental flask diminished can be easily calculated.

For a more complete calculation of the amount of oxygen taken up by the animal in flask A, Draatich added tube (4), the upper end of which is fused into tube (1) between flask A and the middle part of tube (1) (see Fig. 23). The lower end of tube (4) is filled with Hg and ends in a metallic muff with a screw (5) inside it, it may also end in a rubber tube filled with mercury, and provided with a screw clamp. When the experimental animal takes up oxygen, the liquid (2) in tube (1) is shifted to the left towards flask A. By screwing up the screw of the muff (5), a column of mercury rises in tube (4), the gas space becomes compressed and the liquid moves to the right back towards flask B. In this case the volume of mercury which rises in tube (4) is equal to the volume of oxygen taken up by the animal at the given time. The volume is determined from the height of the column of mercury, multiplied by the area of the cross-section of the inner space of the tube (4). Hence, when the inner diameter of tube (4) and the height of mercury column (h) are known, the volume is equal to  $V = \pi r^2 h$ .

Yu. D. Polyakov (1939) suggested several modifications, among them automatic feeding of air into flask A, and a self-recording device which regulates the amount of air fed to flask A. A diagrammatic representation of this apparatus is shown in Fig. 24.

In what follows we shall describe the microrespirograph (as it was called by Polyakov) and its function in the words of the author:

In the Draatich respirometer, mercury is introduced into tube (4) by means of a hand-operated screw, in Polyakov's modification "the lower part of the tube (m) is connected with a metallic socket (P) sealed at one side and with a precise screw thread (c) at the other end. When it is screwed up, the inner volume of the socket decreases, i.e. air enters the tube. The thread is so precise that on closure the device is airtight, even when air is pumped into the socket under pressure of several atmospheres. The screw head is divided into 12 equal parts marked by slanting projections (s). Turning of the screw by one division (i.e. by  $1/12$  of a circle) allows an accurately measured amount of air to enter the socket, and hence to the tube connected with the analyzer. This amount can be determined if the diameter and groove of the screw are known. Nevertheless, the exact amount of air must be

determined with the aid of Fig. In our model, one turn of the screw allows 6 microliters of air to enter the tube. The turn of the screw by one division is accomplished by means of an electric magnet (em) which is attracted by an anchor (an).

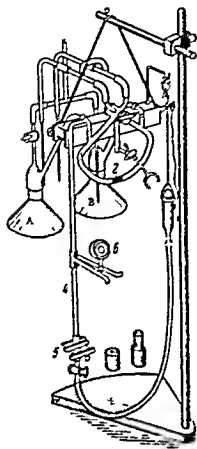


FIGURE 23 Direct respiration

Through the central part of capillary scale of the index (1) passes a narrow and strong beam of light which falls on a photoelectric cell (pe). The narrow and thick-walled empty capillary tube disperses the light strongly, so that the photoelectric cell is weakly illuminated. But when the ray passes through the drop of paraffin the scattering capacity of the capillary tube diminishes, and the photoelectric cell receives a strong beam of light. The photostream passes into an amplifier and a relay (placed in one box-u). The relay causes the electric magnet to switch on and off into the rectifier circuit fed from the main. Alternating current is unsuitable. The increased beam of light can be controlled so that it will be insufficient to close the relay until the paraffin drop is beyond the reach of the beam of light, and permits the closure of the relay when the drop passes across the beam.

The apparatus works in the following manner. The flask analyzer contains the experimental animal which consumes oxygen at a certain rate. Carbonic acid given off during respiration is absorbed by alkali. The paraffin drop in the index scale initially placed to the right of the light beam (i.e. from the analyzer) gradually moves to the left (with decreasing oxygen content) until it passes across the beam of light. The light-scattering capacity of the capillary thus decreases, and the photoelectric cell receives more light, which causes ampli-

fication of the stream of photons. This causes the relay to close, which in turn switches on the electric magnet in the rectifier circuit. The electric magnet attracts the anchor which turns the screw by one division. The turning of the screw by one division lets an exact amount of air into that part of the system which is connected with the analyzer (in our apparatus - 6 microliters of air). As a result the drop of paraffin again moves to the right, i.e. away from the analyzer. The beam of light reaching the photoelectric cell weakens, the stream of photons diminishes, and the relay switches off the electric magnet. Switching on will then take place only when the drop of paraffin passes across the beam of light, i.e. when the experimental animal will have taken up 6 microliters of oxygen.

This will again cause the switching on of the relay, and give a new turn of the screw. This will go on until the end of the screw. Thus, during the time elapsed between two turnings of the screw, the experimental animal had taken up 6 microliters of oxygen. If the time during which 6 microliters of



oxygen had been taken up is known, the rate of oxygen uptake (per hour or minute) can be easily calculated.

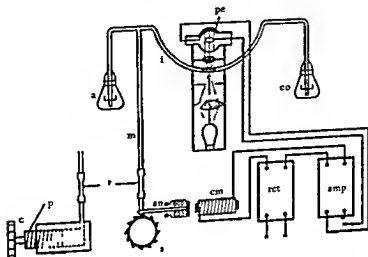


FIGURE 24 Polyakov's modification of Drastich respirometer

1-analyzer (respiration chamber), co compensator T graduated tube, i capillary seals with index (paraffin drop), pe photoelectric cell s-rubber tube, connecting tube T with drum cm amp-amplifier, ret-rectifier, em-electric magnet, an anchor with coiled wire, c-screw p-muffle with thread for feeding air into the tube

The anchor which turns the screw is also connected with a marker which marks a dot on the connected clockwork drum. The greater the distance between two adjacent dots, the longer the interval and the lower the respiration rate of the animal.

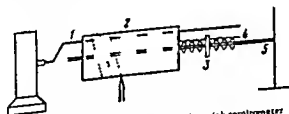


FIGURE 25 Self-registering device in Drastich respirometer (after Yu. D. Polyakov)

1-metallic rod connected with the axis of a clock 2-card-board drum, 3-plate, 4-spring 5 axis of the drum

A schematic representation of the self-recording device is illustrated in Fig. 25. Metallic rod (1) passes through a hole in the wall of a cardboard drum (2) which revolves freely on its axis (5). One end of the rod is connected with an axis of a large hand of a clock. The axis makes a full circle in one hour and so does the drum. Plate 3 is sealed to axis (5). Since this plate is placed between spring spires attached to the back of the drum, it moves the latter along its axis during rotation. Owing to this the drum has two movements: revolving and linear-progressive. The drum is covered with a paper on which the dots are marked. Owing to the dual motion of the drum, the dots are arranged along a spiral. At the end of the experiment the paper is taken off the drum and calculations are made.

In this particular case each dot on the paper corresponds to 6 microliters of oxygen taken up by the animal (the marker drew dots when 6 microliters of air had entered the tube). If the time interval during which this amount of oxygen was taken up is known, the total oxygen uptake may be calculated

as  $\frac{6.60}{t}$  = amount of oxygen taken up (in  $\mu$  l/hour). Or in a general form:

$\frac{n \cdot 60}{t}$  = amount of oxygen taken up, where  $n$  = volume of air introduced at each turn of the screw;  $t$  - time elapsed between two turns of the screw.

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## DETERMINATION OF ACTIVE METABOLISM

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Energy-yielding metabolism is one of the main biochemical criteria organically linked with the main vital functions of animals, including fish. At the present a wealth of empirical data has accumulated on energy-yielding metabolism of fish in a relative resting state. These data enable one to make a number of generalizations on individual taxonomic groups and on the class of fish in general (Vinberg, 1956). The active metabolism of fish (energy expenditure for muscle work) has been studied less thoroughly. This aspect of metabolism, however, is not less important for the solution of numerous theoretical, in particular evolutionary, and of practical problems. Fish in their natural environment never exist in a resting state; for various calculations one must therefore have a certain concept of their energy expenditure during motion at various speeds. The obtaining of these data has been difficult until recently, due to the absence of suitable methods. Only recently, a number of methodological problems have been overcome and data of primary importance were obtained.

Some ideas on the magnitude of energy-yielding metabolism have been provided by experiments with fish in a resting state. Metabolism of fish in motion hampered the assay of standard metabolism. Attempts to estimate the magnitude of this hindrance and to design experiments so as to reduce it to a minimum led to rough and tentative estimates of active metabolism.

As an example, the works of V. S. Ivlev (1938), Keys, Webb and Fuller (cited by Weinberg, 1956), may be consulted.

Studies of Spoor (1946) were devoted to the relation between oxygen consumption and fish activity. This author used the following method.

The main part of the Spoor apparatus is a thermostat with a vessel for the experimental animal (Figure 1). The vessel is provided with a system of tubes with the aid of which a constant flow of water is ensured. The respiration vessel is filled with water from the left vessel (Figure 1, 11) in which a constant level, and hence a constant rate of water replacement, is maintained. On the right side of Figure 1 there are tubes which serve for the outflow of water. One of these serves for stabilizing the water level in the respiration vessel, and the other for filling of beakers in which determinations of oxygen content are carried out.

The distinction of this apparatus lies in that the respirometer is provided with a sensitive mechanism which registers the ripples of water caused by fish movement. This device consists of blades whose position

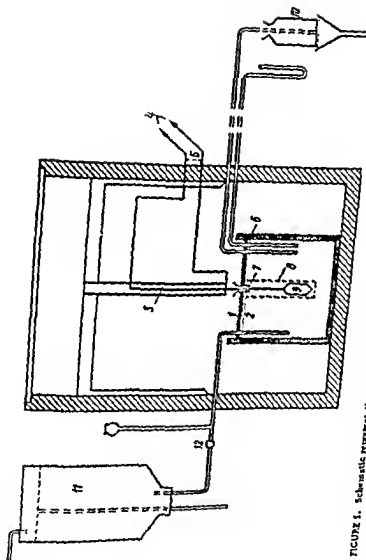


FIGURE 1. Schematic representation of poor apparatus  
 1—paraffin oil; 2—glass plate; 3—copper wire; 4—insulative wire; 5—resistance; 6—screen (paraffin oil and glass)  
 7—glass tube (trailing box); 8—wire and protective blade; 9—blade for registration of fish movement; 10—  
 beaker for withdrawal of water samples; 11—water vessel; 12—stopcock.

in water changes when a ripple appears, and transmit the corresponding impulses through a relay of high sensitivity to an automatic self-registering device (this is not shown in the Figure)

It was seen that various external factors (even the presence of a person in the room) affect the behavior of fish, their movements become more marked, and as a result, their metabolic rate increases. To eliminate these obstacles, the tubes and electric wires were continued to adjacent rooms and the apparatus was used as if by remote control.

The Spoor apparatus enables one to carry out experiments for indefinite periods of time, some experiments of this author lasted several months.

The main task of Spoor was not to study the active metabolic rate, but to find the real value of basal metabolism which could not be measured in the conventional respirometers. This apparatus is therefore so designed that it can register the number of movements, but cannot assay the magnitude of muscle work performed. This feature led to the creation of other models allowing for the determination of muscle work and metabolic rate.

The apparatus to be described was constructed by Black, Fry and Scott (1939), and has been widely used by Fry and Hart (1948) in a modified form. The cross-section of this apparatus is presented in Figure 2.

The fish to be studied is placed in a ring-shaped vessel with a rectangular cross-section. The outer wall is made of glass, and the inner wall and bottom of metal. The outer diameter of the chamber is 30 cm, its width 7.5 cm, and its depth 15 cm.

The chamber is filled with a known volume of water, the fish is placed inside the chamber and the latter is then closed by a nonhermetical floating lid. The chamber is set in revolving motion with the aid of a special motor. The number of revolutions per unit of time can be controlled. The fish endeavors to remain in its place, and begins to swim against the motion. If the velocity of the latter is known, the speed of the motion of the fish (i.e., work done by the fish) can be easily calculated. 100 ml water samples are withdrawn by means of a pipet through a hole in the lid. The lid is lowered a little for this purpose. The samples are placed in 50 ml beakers, oxygen content is determined by the method of Winkler.

Since the floating lid does not completely isolate the water inside the chamber from the hemisphere, the following additional corrections must be done. The chamber is filled with boiled nitrogen-saturated water in the chamber with a varying oxygen content. The increase in oxygen content of the water in the chamber with the lid in position was then determined. On the basis of these determinations, the calculations on fish respiration were corrected for.

The desired temperature in the chamber was maintained by sprinkling the latter with a stream of water at the desired temperature through tube 6. According to the authors, the apparatus gave reproducible results.

A more complicated and improved apparatus based on similar principles was described by Basu (1959) from Fry's laboratory (Canada). The respiration chamber (Figure 3) is placed on a revolving table. Size of chamber: outer diameter 30.5 cm, inner diameter 11.4 cm, diameter of cross-section 8.9 cm, volume 5,000 ml. The special feature of this apparatus consists in the paired electrodes which induce the fish to stay fixed in one position during motion of the chamber. In other words, electric screens of low potential are created in front and behind the fish (3 volts on electrodes), which do not enable the animal to move in the chamber. The

electrodes are connected with the contacts of the synchronously revolving collector, with the aid of which alternating pairs of electrodes are switched on. The optimal rate is 20 revolutions per minute. The duration of each experiment is 15-20 minutes.

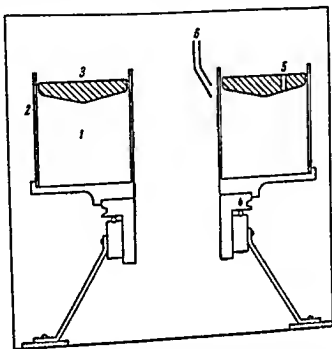


FIGURE 2 Cross section through Fry and Hart apparatus

1—ring-shaped chamber for experimental fish 2—glass wall 3—floating lid 4—movable bottom on bearings with a groove for transmission belt from the motor 5—hole in the lid for withdrawing samples 6—tube for sprinkling the chamber with water of desired temperature

There is always a danger that in spite of the location of electrodes and fields of low tension, cross electric fields may appear which will excite the fish, producing exaggerated movements in relation to movement of the chamber. Data on oxygen consumption as presented by Basu indicated that they depended only little on the rate of linear motion of the fish. This strange circumstance calls for caution in using electric stimulants, in any case in the apparatus constructed by Basu.

The Basu apparatus is represented in Figure 4. The respiration chamber and additional devices permitting the determination of active metabolism at a given partial pressure of  $O_2$  and  $CO_2$  are illustrated.

The Wohlschlag apparatus (Wohlschlag, 1957) is based on a similar principle, but differs in technical details. The ring-shaped chamber is made of transparent plastic, size of chamber: outer diameter 45 cm, inner diameter 25 cm, depth 10 cm. In other words, the cross-section of the chamber is a  $10 \times 10$  cm<sup>2</sup>. The volume of the chamber at 10°C is 10,400 ml. Water samples are withdrawn through a rubber tube provided with a clamp. Water supplies are replaced by water coming from a

bottomless bottle placed above the chamber with neck of the bottle down. The chamber is connected to a revolving mechanism through a thick brass wire (as a revolving mechanism, a hand-operated spring motor of a phonograph may be used). The number of revolutions per minute may be regulated. The entire system is suspended on a portable four-legged table of derrick-cranetype. The length of each table leg, i.e., the height of the apparatus, is 3 m. With the aid of a system of pulleys the motor with the chamber may be rapidly lowered or lifted.

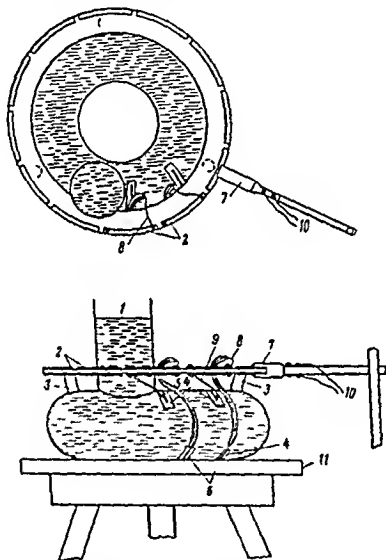


FIGURE 1. Revolving respiration chamber of Bata. Top—cross-section bottom—lateral view.

1—tube over the hole (height 12.7 cm. diameter 8.9 cm.) 2—commutator 3—probe 4—aluminum electrodes 5—upper electrodes 6—lower electrodes 7—conductors connecting lower plates of commutator with upper electrodes 8—conductors in plastic tubes which connect upper plates of commutator with lower electrodes 9—pair of contact copper brushes 10—connection between brushes and the metal 11—revolving table.



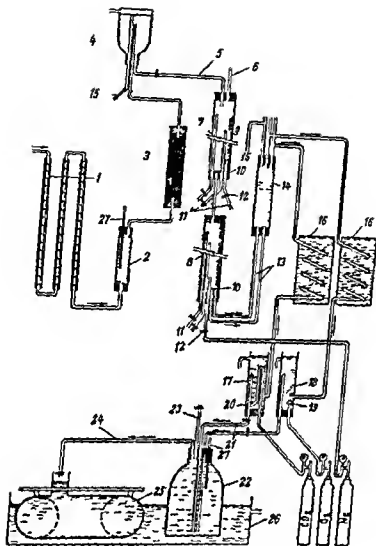


FIGURE 4 A device for continuous supply of water at varying temperature and varying  $\text{CO}_2$  and  $\text{O}_2$  contents in the Bass apparatus

1—electric (pyrex glass) heater 5 000 watt (water flows from stopcock); 2—vessel for measuring water temperature; 3—filter with glass wool 4—reservoir with constant level 5—inlet into a spraying column 6—gas outlet 7 and 8—oxygen spraying columns 9—glass spheres 10—aluminol protective plate 11—inlet for nitrogen 12—screw clamps 13—inlet into 14 14—constant level reservoir; 15—overflow of water excess 16—heating vessels 17—carbonic acid equilibrium column 18—oxygen equilibrium column, 19—inlet for oxygen, 20—inlet for  $\text{CO}_2$  21—entry into 22 22—mixing bottle 23—air trap 24—inlet 25—respiration chamber 26—constant temperature water bath 27—thermometer.

The apparatus is so constructed that it can be used under natural conditions. It is placed in a natural water reservoir and the chamber is immersed to a depth of somewhat less than 1 m. The natural conditions of the fish in question (temperature, light) are thus maintained (the fish used

in these experiments are taken from the reservoir in which the chamber is immersed.

The study is carried out as follows: The apparatus is assembled, the chamber is filled with water from the reservoir in question, the fish is caught and immediately placed in the chamber. The lid is closed, the chamber is immersed in water and set in revolving motion. The size of the chamber is such as to correspond to 1 m distance passed by the fish during one revolution.

Water samples are withdrawn after 10-15, 30, 45 and 60 minutes. Water temperature is measured at the same time. For each sample 265 ml of water are withdrawn, the loss being immediately replaced from the reservoir. This procedure takes 1 minute (the chamber must be taken out of the water and the motor must be stopped). Oxygen is determined by the simplified method of Winkler.

I have somewhat modified (Ivlev, 1962) Wohlschlag's apparatus so that it can be used for studies on small fish fry under laboratory conditions. A schematic representation of our modified apparatus is depicted in Figure 5.

The revolving chamber is connected to a reductor which allows for control of the rate of revolutions. The reductor in its turn is connected with a Warren electric motor with a constant number of revolutions. In our apparatus no additional water reservoir is present, so that only two oxygen concentrations in the chamber can be registered before the experiment, and after the experiment (after 20 to 60 minutes). Studies on active metabolism of salmon and bleak fry carried out with the aid of our apparatus gave reproducible results.

In the U.S.S.R., in the Institute of Physics of the Sea, an apparatus has been constructed for studies on active fish metabolism. This apparatus is based on a different principle (Kovalevskaya, 1956). In this apparatus (Figure 6) the respiration chamber is an immobile, closed, O-shaped vessel. The motion of water which the fish must overcome is created by a screw propeller (moved by an electric motor). The number of revolutions of the latter is regulated by means of a rheostat. The axis of the motor and the screw propeller are connected to a tachymeter (calibrated according to the flow of water in the chamber with the aid of a TsAGI flow meter).

In the upper part of the chamber there is a rectangular opening through which the fish to be studied is placed in the chamber. Water inside the chamber is isolated from the atmospheric air by a water-filled receiver placed above the opening. The receiver serves also as a reservoir from which water is taken to replace water withdrawn for the analysis. Water is withdrawn through a special stopcock.

The chamber is transparent; its length is 100 cm and its volume 24,000 ml.

It is essential that the fish should be placed in the chamber between two nets; the first net (with the stream of the water) is provided with special openings which facilitate a uniform distribution of water currents inside the section in which the fish is placed. The general appearance of this apparatus is depicted in Figure 7\*.

In her work with this apparatus, L. A. Kovalevskaya made several methodological and fundamental errors, which were reviewed by G. G. Vinberg (1956). These errors, however, have nothing to do with the

\* The photograph has kindly been provided by K. D. Alakseeva, who at present works with this apparatus

apparatus itself, and it can be assumed that work with this apparatus or any other model constructed on a similar principle will yield reproducible and reliable results

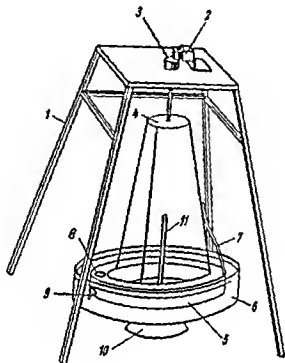


FIGURE 5 Wobacklag apparatus as modified by Ivlev

1—support tripod 2—warren electric motor with a constant number of revolutions (60 rpm) 3—pulley with three speeds (for revolving the respiration chamber at 0.65 2.01 and 6.42 cm/sec) 4—disc with suspension brackets 5—respiration chamber from plexiglass (outer diameter 24 cm inner diameter 16 cm cross-section 4x4 cm volume about 1000 ml) 6—constant temperature water bath 7—tube connecting respiration chamber with the atmospheric air 8—lid covered opening (through which the fish is placed in the chamber) 9—stopcock for withdrawing water samples 10—prop for water bath (removed during sampling) 11—thermometer paper strip with transverse black bands which surrounds the water bath and promotes fixation of the fish in one place during the revolving procedure is not shown.

Studies on active metabolism are of great scientific and practical interest. Let us recall that the rate of fish respiration obtained by conventional methods is not representative of the level of basal metabolism, because the fish during the experiments is never in a state of complete rest. As a matter of fact the metabolic rate determined is called an ordinary or standard metabolism. Attempts made to ascertain how much higher this standard metabolism is than the basal metabolism have shown that the

latter constitutes about 60% of the standard metabolism which is empirically determined (Vinberg 1956) This value has been obtained on the basis of sporadic calculations carried out by physiologists It can be assumed that this value shows wide variations, depending on the age, state and species features of the fish studied as well as on the technique employed by each worker

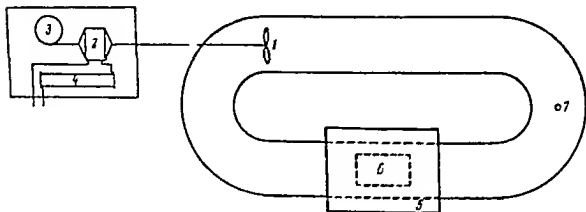


FIGURE 6 Schematic representation of Kovalevskaya's apparatus

1—screw propeller 2 electric motor 3—tachymeter 4 rheostat 5—vessel filled with water for closing the opening, 6 stopcock for withdrawing water samples

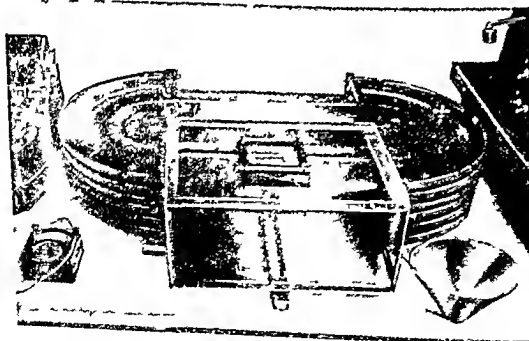


FIGURE 7 General view of Kovalevskaya's apparatus Behind the box the propeller for creating water flow in the chamber can be seen

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## BALANCE SHEET EXPERIMENTS ON NITROGEN METABOLISM OF FISH

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Balance sheet experiments on nitrogen metabolism are widely used in physiological and ecological studies. A knowledge of nitrogen balance is indispensable for the determination of protein requirement in the animal in question. The amount of nitrogen intake of the animal with the food, as well as that of nitrogen excreted (with urine, sweat and feces) is measured. Nitrogen balance sheet experiments with fish differ from those carried out with warm-blooded animals. In experiments with warm-blooded animals the observations (from the beginning to the end) are made on one animal. In the case of fish, however, numerous specimens may be used in a single experiment. In addition, cattle-breeders are interested in the amount of nitrogen deposited in the body of the cattle, this value represents the difference between the amount of nitrogen taken in with food and the amount excreted in the feces and in products of protein metabolism.

In experiments with fish the unknown value is usually the amount of nitrogen intake by the fish with the food per time unit. This is equal to the sum of nitrogen deposited in the body of the fish and the nitrogen excreted in the feces, and other products of protein metabolism. The amount of nitrogen deposited is determined by the difference between the average nitrogen content in the body of the fish at the beginning and end of the experiment. Nitrogen content in the fish body can be determined after killing all the fish under study. The amount of nitrogen which has not been utilized and nitrogen in the products of protein metabolism is determined experimentally as in the case of higher animals.

Relationship between the amount of nitrogen utilized and the weight of food eaten is determined by analysis of the content of fish intestines. The analyses are carried out at the beginning and end of the observation, and show the average weight ratio between various food components and their average nitrogen content. Hence the amount of nitrogen utilized per weight of food eaten can be easily calculated.

From these facts it follows that the accuracy of the results obtained is to a considerable extent determined by the accuracy with which the gain in weight of fish is determined. The gain in weight depends both on the fish species and the duration of the experiment. Hence the duration of the experiment is of paramount importance.

To determine the increase in nitrogen content in the body of the fish, the experiments on nitrogen balance should be carried out for relatively long periods of time

It is difficult to determine the extent of increase in nitrogen in short-term experiments, when, on the other hand, the experiments are too long, the features of growth at the varying growth stages remain unobserved. Hence the duration of the experiment should depend both on the growth features of the object to be studied and on the experimental conditions.

The highest rate of growth of fish is observed in the early stages of their development. With the growth of the fish the rate of growth diminishes, although it never ceases altogether. Hence the duration of experiments with fish fry should be shorter than that of experiments with adult fish. Studies on fry of rapidly growing fish (sturgeons, various predatory fish, sazan, carp and others) commencing from the stage of active feeding until they attain an average weight of 0.5 g should last 5 days. In experiments with fish with a lower rate of growth (bream, roach) observations should be carried out when their average weight is 0.2 g, and in experiments with such fish as "moderlieschen", when their average weight is 0.1 g. With further growth, observations may be made every 10 days. In the case of adult fish under constant environmental conditions, except for spawning periods, determinations may be made every 15 days. In summer the interval between the determinations should not exceed 15 days. This is because the metabolic rate depends not only on the age of the fish, but on the food resources, temperature, amount and composition of the gases dissolved in the water, and other environmental conditions, which in summer may show a marked change.

If the fish are kept in an aquarium or in ponds, and their growth conditions are changed (change of food etc) the experiments on determination of the amount of nitrogen excreted should be carried out twice (at the end of the preceding period of growth and immediately after changing growth conditions).

In winter when the metabolic rate is lowered and the hydrological conditions are relatively stable, the determinations (in case of both adult and young fish) may be carried out every 25 - 30 days

In experiments with spawning fish, the observations should be carried out before the spawning and immediately after its completion. To begin experiments during spawning is purposeless, since excretion of sexual products will distort the estimate of nitrogen metabolism. The characteristic property of fish during spawning is their high rate of protein deamination. It must be stressed that the methods of study on nitrogen metabolism in fish during spawning have not yet been adequately elaborated, and must be the subject for specially designed additional studies.

Until now we were concerned with experiments on fish from freshwater reservoirs with rapidly changing hydrological and food conditions. In the sea these changes are slower and more gradual. Hence balance sheet experiments with marine fishes can be carried out for longer periods of time, and the experiments can be adopted to the various stages of fish development.

#### COLLECTION OF MATERIAL

Healthy intact fish should be used. Fry in early stages of development are taken out of their habitation places by means of ordinary landing-nets

made of fine-meshed gauze or netting. The fry are taken out of the landing-net (which should not be taken out of water) into a beaker or a small net and are transferred into a bucket. Older fry may be caught by means of a fine-mesh acrafer, and adult fish by means of sweep-nets. The catch is selected without taking the landing-net out of water. Small tender fry (bream and roach fry, for example) is transferred into a bucket by means of a special net. Less delicate fry, such as wild carp fry and adult fish are better transferred by hand. Fish caught in nets are unsuitable for the experiments, since the nets tend to do damage to the fish caught in them. Fish caught by traps, dragnets and sweep-nets can be used only if the time spent by the fish in these traps is known. Fish caught in sweep-nets can be used in the experiments if they are caught during repeated sortings carried out at intervals not longer than one hour.

The accuracy with which the increase in nitrogen content is determined depends on the accurate determination of the weight of the fish studied. Since the growth rate of any single specimen in the group studied (each group consists of fish of the same age) fluctuates, considerably large numbers of specimens should be subjected to analysis. The greater the fluctuations in the weight of individual specimens, the more fish should be subjected to analysis for the determination of an average gain in weight and size.

It is desirable that in experiments with fish under natural conditions not less than 50 to 100 specimens should be weighed and measured for each determination. In experiments carried out in the sea, determination of the average weight of fish is complicated by the presence of several populations differing from each other in their growth rate. Since it is very difficult to carry out long-term experiments with a single fish population, the average weight of the fish should be determined on the basis of several samples taken at various points of the water reservoir studied.

The length of the fish is measured on a special graduated board. The accuracy of measurement is up to 1.0 mm. Young fish in early stages of development are weighed on a torsion balance with an accuracy of 1 mg, and fry are weighed on an analytical balance with an accuracy of 10 mg. Adult fish are weighed on ordinary Roberval scales. Before being weighed, the fish should be slightly dried on a filter paper or wiped with gauze (to remove excess moisture). The average weight of fry may be determined by weighing all fry together. (From 5 to 15 weighed fish were used in studies on nutrition.) Fry is fixed in a 4% formaldehyde solution. In large fish only intestines are examined (the date, place of capture, size, weight, sex, stage of ripeness of the sexual products and age should be recorded). It is desirable that all these measurements should be made prior to the experiment proper (i. e. to select fish for the experiments on the basis of these measurements). If the investigator has a small number of fish at his disposal (for example fish grown in an aquarium) the balance-sheet experiments are carried out first and then the remaining fish are measured and weighed (to ascertain the average body size and weight).

Fry in early stages of development react very poorly to the procedures of the experiment and body measurements, and usually die. Subadult fish, however (young wild carps, carps and sturgeons, for example), if properly handled survive weighing and after the experiment may be transferred back into the water reservoir or aquarium from which they were taken.



Before commencing balance-sheet experiments, one must prepare vessels in which the fish will be placed and provide adequate amount of filtered water. Broad, flat-bottomed vessels should be used. Water in such vessels should be well aerated and excreta can be easily removed. For experiments with fish fry glass vessels or other suitable vessels may be used. For experiments with larger fish, aquarium jars or enamel basins may be used. For experiments with fresh-water fish, aluminum tanks and galvanized iron tubs may be used. To facilitate the withdrawal of excreta, the glass vessels should be placed against a white background (on a white oil-cloth, for example) while the basins should be coated on the inner surface with white enamel. The vessels should be protected from sunlight, care should be taken that the fish will not be disturbed during the experiment. To avoid contamination and to prevent the fish from jumping out of the vessel, the latter should be covered with glass or gauze.

Three vessels are prepared for each experiment: two for the experiment proper and the third to serve as a control. In addition, a vessel with reserve fish should be ready in case the investigator wants to change the fish in the course of the experiment.

The aquarium is filled with water from the reservoir from which the experimental fish had been taken. Before use, water should be filtered through filter paper or cotton wool. Tap water may be used only for experiments with fish grown in tap water. Chlorine-free water should be used. The filtered water is kept in one large vessel from which it is distributed to the experimental vessels.

The amount of water poured into the aquarium should be calculated so that the fish will not suffer from lack of oxygen during the three-hour experiment. Water should not be added in excess, since in such a case the metabolite products excreted will be highly diluted and the analytical results distorted. It is difficult to determine the accurate ratio of water to the number of fish used in the experiment. This depends on the fish species, their size, environmental conditions (water temperature, oxygen content in water) and on the number of fish used in the experiment and volume of the vessels used. Salmon, for example, which are very sensitive to oxygen content the water, should be taken in fewer numbers per given volume of water than sazan and carps which can endure low oxygen concentrations more easily. If the water temperature rises, two approaches are feasible: 1) to increase the volume of water while the number of fish remains constant, or 2) to decrease the number of fish used in the experiment while the volume of water remains constant.

It is easy to judge whether the experimental fish suffer from lack of oxygen. If the concentration of oxygen is adequate the fish swim about calmly. When the concentration of oxygen becomes critical, the fish begin to make gulping movements. In this case fresh water should be added to the aquarium. The larger the number of fish used in the experiment, the more accurate the results. The obtaining of large number of fish, and especially of fry in their early stages of development, may be especially difficult. Two hundred ml samples are needed for two parallel determinations of nitrogen content in water, hence the volume of water in each aquarium should not be less than 250 ml. This quantity of water is enough for experiments with 30 - 50

actively feeding sazan fry. For experiments with fish weighing up to 1.0 g the vessel should be filled with 500 ml of water, and for experiments with larger fish, 1000 ml of water should be used. Experiments with single fish should be avoided. Experiments with single fish are carried out only with large specimens and in cases when it is difficult to obtain fish in the necessary numbers, or when appropriate vessels for studies on large fish are lacking. The amount of water in the experimental vessel must be accurately measured.

The temperature of water in the experimental vessel should be as close as possible to that of the water reservoir from which the fish is taken. If the difference is more than 2 °C, the experimental vessels should be placed in a water bath with a desired temperature. Large aquariums or tubs may be immersed in water reservoirs from which the fish had been taken. This should be done near the shore of the water reservoir, in places protected against waves.

Excreta should be collected in weighing bottles, the former are taken out of the aquarium by means of Pasteur pipets provided with rubber bulbs. The diameter of the pipets should be such as to allow removal of excreta. The excrements should be taken together with a minimal amount of water.

Water from each aquarium is poured into a separate bottle (500 ml bottles with rubber stoppers or with ground joints and glass stoppers should be used).

For fixation of the water samples a small amount of chemically pure sulfuric acid and chloroform should be prepared beforehand. Before being weighed, the fish should be wrapped in gauze or filter paper to dry them, hence filter paper or gauze should be prepared beforehand. A measuring board with 1 mm graduated scale should also be prepared. Finally, appropriate scales depending on the size of the fish, as well as boxes in which small fish or stuffings from large fish are dried, should be kept ready.

The fish to be studied should be brought immediately to the laboratory. They are rinsed with fresh water to remove slime and dirt attached to them, and placed in the aquarium. Small fish are transferred into the aquarium by means of small landing nets, and large ones by hand. The time as well as the water temperature are recorded.

Damaged specimens and those with incomplete scales are unsuitable. In order not to disturb the fish they are neither weighed nor measured before the experiment. Selection of fish with average weight and size is done by eye. Experiments with fish of different size and weight should be avoided, because the metabolic rate of such fish will differ from specimen to specimen. During the entire experiment the fish must be under constant observation.

Fish excreta should be removed immediately from the aquarium. Excreta left in water for a length of time will lose considerable amounts of nitrogen. The excreta will be dispersed by the swimming fish and will then be difficult to remove from the aquarium. Inadequate removal of excreta will not change the results of experiments on nitrogen balance, but will give a distorted representation of non-utilized nitrogen and nitrogen from protein metabolism. Excreta from each aquarium are collected in separate weighing bottles. If there are little excreta, they may be collected from all aquariums and placed in one weighing bottle.

Each experiment usually lasts three hours. Termination of the experiment should be recorded. The fish are removed from the aquarium, dried

on a filter paper or wiped off with gauze and then weighed and measured. Small fry is weighed with an accuracy to 1 mg, larger fry with an accuracy to 10 mg, and fish weighing 1 kg or more with an accuracy to 10 g. Fish of similar size may be weighed together.

From each aquarium 250 to 500 ml of water are withdrawn including the control one, and poured into bottles specially prepared for this purpose. Ammonia present in the water samples is bound by adding 0.5 to 1.0 ml of chemically pure sulfuric acid.

If the samples are to be stored for a prolonged time, fungus growth is prevented by adding several drops of chloroform. The bottles are then tightly stoppered with rubber plugs.

To obtain data which would represent the average daily nitrogen excretion the experiment must be repeated four times at different periods of the day - in the morning, at midday, in the evening, and at night, because the rate of excretion of products of protein metabolism varies, depending on the time of day.

If in the course of the experiment some fish seem to suffer discomfort, they should be replaced by reserve fish of similar size. To prevent oxygen starvation, the oxygen content of water should be determined before the experiment, and the number of fish placed in one aquarium should be correlated with their respiration rate. If in the course of the experiment the fish show signs of oxygen deficiency, fresh water should be added, if the size of the vessel does not permit it, the duration of the experiment should be shortened. If any abnormal behavior of the fish studied has not been noticed in time, the entire experiment should be discarded.

If fry in the early stages of development excrete negligible amounts of feces, the latter may be left uncollected. In this case, nitrogen in the excreta is determined together with nitrogen of the products of protein metabolism. To this end the fish are removed from the aquarium at the end of the experiment, and the excreta suspended uniformly in water by shaking and pipetization.

The excreta are collected in special weighing bottles and dried in a thermostat at 70°C. In this state they are kept until analyzed. For determination of the increment in nitrogen in the group studied, the per cent content of nitrogen in the body of the fish is determined at the beginning of the experiment and at several intervals. For this purpose, both experimental fish and those employed for measuring may be used. It is important that all fish of the given group should be of similar average size. To avoid possible individual variations, each sample to be analyzed should consist of several specimens. In experiments with fry in the early phases of development several tens of specimens should be used for each analysis (the total weight should not be less than 0.5 g). For experiments with older fry and larger fish, 4-5 specimens should be used for each determination. Very large fish are minced and the sample is taken from the homogenate. If only nitrogen is to be determined, samples weighing 2-4 g are sufficient. If analysis of other substances is contemplated, samples weighing 10 g and more should be used.

The samples (fish or fish homogenates) are weighed accurately (in glass weighing bottles) and dried in a thermostat at 70°C (air-dried samples). Air-dried samples may be stored for a prolonged period. Fish and fish homogenates may be fixed in strong sulfuric acid in wide-necked pycnometers with

ground joints and glass stoppers, 3-5 ml of strong chemically pure sulfuric acid are added to each pyknometer, depending on the weight of the sample.

All balance sheet experiments should be carefully recorded. Protocols should include the date of the experiment, the volume of water in the aquarium, water temperature in the aquarium at the beginning and end of the experiment, the exact time of the beginning and end of each experiment, the number of weighing bottles in which the material was collected from each vessel, and their weight, number of beakers with water samples carefully labeled to show from which aquarium they came, the weight of the fish or homogenate and the number of the weighing bottle or pyknometer in which it is placed. In addition, the experimental conditions, method of collecting of the fish and their general well-being during the experiment should be recorded.

#### CHEMICAL ANALYSIS

The nitrogen content in the digested fish body, water and feces is determined by the micro-Kjeldahl method. The substance is digested by boiling with concentrated sulfuric acid in the presence of a catalyst. Protein is decomposed and organic nitrogen is converted into ammonium sulfate. After addition of excess alkali the  $\text{NH}_3$  liberated is steam-distilled in the micro-Kjeldahl apparatus and trapped in an excess of 0.01N solution of sulfuric acid. Acid excess is back-titrated with alkali solution. Thus the ammonia (and hence nitrogen) content can be determined. The following reagents are needed:

1. Concentrated, chemically pure sulfuric acid (sp. gr. 1.84).
2. Working solution of 0.01 N sulfuric or hydrochloric acid are prepared from stock solutions. A 0.1 N solution of the corresponding acid is first prepared by diluting concentrated acids in ammonia-free water in 1000 ml volumetric flask; 0.01 N solutions are prepared from 0.1 N solutions by ten-fold dilution. Growth of fungi in these solutions can be prevented by adding several drops of chloroform.
3. Working solution of 0.01 N solution of alkali is prepared by appropriate dilution of chemically pure sodium hydroxide in  $\text{CO}_2$ -free water.  $\text{CO}_2$  is removed by boiling; 0.4 g of NaOH is dissolved in 1000 ml of distilled water. Carbonates are removed by adding several drops of barium chloride ( $\text{BaCl}_2$ ), which precipitates carbonates in the form of barium carbonate. The addition of barium chloride does not interfere with the analysis.
4. Concentration of 0.01 N solutions of acids and alkali is determined by titration with an accurately prepared 0.01 N solution of borax -  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ . For this purpose  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$  is recrystallized twice and 1.907 g of the salt dissolved in 1000 ml of ammonia-free distilled water; 5 ml of this solution (measured with the aid of a volumetric pipet) with 2 drops of Tashiro indicator are transferred into a conical flask and titrated with the working solution of hydrochloric or sulfuric acid. Addition of the indicator turns the borax solution green. In the course of titration the solution becomes colorless and then assumes a pink color. The end-point is indicated by the appearance of a stable pink color.

From the volume of 0.01 N acid solution used up for the titration of borax the normality of the former is calculated. Correction is made to the third decimal point. Multiplying the amount of acid solution used up for the titration by the correction, the exact normality of the working solution of the acid can be calculated.

For determination of the exact normality of 0.01 N alkali solution, 5 ml of standard solution of acid is titrated with the alkali solution in the presence of the same indicator. The endpoint is indicated by the appearance of a stable green color. The correction for alkali solution is calculated from the ratio of the amount of acid used for titration of the alkali. Since the concentrations of alkali and acid solutions change with time, their normality should be checked periodically.

5. Tashiro Indicator is prepared by dissolving 0.2 g of methyl red in 100 ml of 96% alcohol and 0.1 g of methylene blue in 100 ml of 96% of alcohol. Both solutions are kept separately and mixed in small portions (v/v) before used. The color of the indicator mixture in an acid medium is violet and in alkaline medium it is green.

6. 33% solution of sodium hydroxide. For the preparation of the latter 330 g of NaOH are weighed and dissolved in more than 1000 ml of distilled water. The solution obtained should be boiled until its volume is 1000 ml. For the preparation of this reagent no great accuracy is needed.

7. Crystalline cupric sulfate ( $\text{CuSO}_4$ ) - catalyst.

FIGURE Micro Kjeldahl apparatus for distillation of nitrogen

1-distillation flask 2 condenser, 3 branching of tube 12 of the distillation flask 4 intermediary receiver of water vapor 5 glass tube inserted into the steam generating vessel 6-condenser 7-steam generating flask, 8 gas-funnel 9-receiver, 10 11 glass tube, 12 tube of distillation flask, 13 glass tube

Micro-Kjeldahl distillation apparatus (depicted in the Figure) consists of a 2000 ml steam-generating flask 7, and intermediary steam receiver 4, distillation flask 1 with funnel, condenser 6 and receiver 9.

Distilled water in flask 7 is boiled until water vapors are formed (the flask is heated on an electric heater), 10 ml of water should be converted into vapor in one minute. Water in the flask should have an acid pH, which is obtained by adding several drops of phosphoric or sulfuric acid. The acid pH is shown by an appropriate indicator. Uniform boiling is secured by several capillary tubes sealed at one end, or with glass stones. The flask may be refilled without the necessity to dismantle the whole apparatus, by pouring water through tube 5, which is connected with a rubber tube and funnel 8.

Flask 7 is filled with water, and tube 5 is closed with a clamp at place where it connects with funnel 8. Water vapor generated in the flask passes through vessel 4 and into the distillation flask 1. Glass tubes 11 and 13 should be connected through a rubber tubing, so that the distance between the two glass tubes will be 15-20 mm. The inner diameter of tube 12 of the distillation flask (through this tube the sample to be analyzed is poured into the distillation flask) should be 5 mm, and its branch 3 through which the sample to be

analyzed is poured in should be a capillary with an inner diameter of 2 mm. The site where tube 3 and 12 are connected with each other should be somewhat distended. The small funnel 8 is connected with tube 3 by means of a rubber tube. The rubber tube should be soft, the distance between the funnel and tube 3 should be at least 15 mm, here the rubber tube is closed by a clamp. The end of tube 12 should be from away from the bottom of the distillation flask. The diameter of the distillation flask should be 55 mm. In its upper part the distillation flask is tightly connected with a condenser by means of a rubber tube, the straight part of this tube should be 60 cm long, and the length of the condenser 40-45 cm. The rate of flow of water through the condenser should be regulated so that the temperature of water during condensation will be around 50-60°C.

Nitrogen content in the body of the fish is determined in the following way. The sample is dried in a thermostat at 70°C to constant weight, and transferred into a desiccator. The difference between wet and dry weight indicates the content of moisture which is expressed in per cent of the wet weight. The sample is carefully ground and again dried to constant weight. A hundred mg samples in duplicate are placed in 50-100 ml Kjeldahl flasks. The weight of the sample is determined in this way: the weighing bottle with the sample is weighed on an analytical balance, a portion of the sample is placed in the Kjeldahl flask and the weighing bottle is reweighed. The difference in weight between the first and second weighing shows the weight of the sample to be analyzed. The sample is placed in a Kjeldahl flask by means of a spatula. The sample should not adhere to the flask walls. Five ml of concentrated sulfuric acid and several granules of selenium or  $\text{CuSO}_4$  (catalysts) are then added to each Kjeldahl flask. The flasks are closed with glass bulbs (cold fingers) and placed on a gas burner or electric oven. The sample is digested in a hood, since after water is boiled off  $\text{SO}_2$  fumes appear. From time to time the Kjeldahl flask should be removed from the burner and carefully shaken to remove undigested particles which may adhere to the flask wall. As digestion proceeds, charring occurs and then the solution clears and becomes colorless. The sample is considered digested when after cooling it is completely transparent or slightly bluish due to the pressure of  $\text{CuSO}_4$ . Even a slight yellowish tinge indicates that the digestion is not complete. A control flask with 5 ml of sulfuric acid and several crystals of  $\text{CuSO}_4$  is also subjected to digestion (control for the determination of N content in  $\text{H}_2\text{SO}_4$ ).

The digested samples are poured into 200-250 ml volumetric flasks. The Kjeldahl flask is rinsed with several portions of ammonia-free distilled water, and all washings are combined in the volumetric flask. The volume is made up with ammonia-free distilled water. Ammonia-free water is prepared from distilled water by evaporating the latter to 1/3 of its initial volume. The content of the volumetric flask is mixed, and 2-3 ml aliquots are withdrawn (by means of a volumetric pipet) and transferred into the micro-Kjeldahl distillation apparatus. Here ammonia is released by adding strong alkali and then trapped in the receiver in a 0.01 N solution of sulfuric or hydrochloric acid. Five to ten ml of the above acid and several drops of Tashiro indicator are placed in the receiver. Distillation of the control flask is considered complete when the volume of the distillate is equal to the volume of the sample and the reagents added. Completion of ammonia distillation is tested by means of litmus paper, Congo (filter) paper or the Nessler reaction. Yellow color indicates the presence of ammonia. If the sample remains colorless, distillation is complete. If in the course of distillation the

violet color of the sample turns colorless or green, this indicates that the amount of acid in the receiver was not sufficient to bind the entire amount of ammonia distilled. In this case the entire procedure should be repeated, and the amount of acid in the receiver should be correspondingly increased.

The distillate is titrated with exactly 0.01 N alkali solution. The violet color disappears, and the sample becomes colorless and then green. The endpoint is reached when the sample assumes a stable green color. The 0.01 N solution of acid in the receiver and the 0.01 N solution of alkali for titration are measured exactly by means of a microburette with an accuracy to 0.01 ml.

Each sample is distilled in duplicates. If the results differ by more than 0.05 ml, distillation should be repeated.

The difference between the amount of standard solution of the acid in the receiver and the amount of standard solution of alkali used for titration is equal to the amount of acid bound by the distilled ammonia. Since 1 ml of a standard 0.01 N solution of sulfuric acid binds 0.14 mg of nitrogen, the nitrogen content in the distillate and in the entire sample can be calculated. The nitrogen content of the sample should be corrected for the amount of nitrogen present in sulfuric acid, as shown by the control sample which was subjected to the same procedure as the sample. Nitrogen content is usually expressed in per cent of the weight of the sample (weight per cent). For this purpose the value obtained is divided by the weight of the sample and multiplied by 100. More accurate results are obtained when an average content of two samples is taken. If the nitrogen content of two samples differs by more than 0.2% a third sample should be analyzed.

To ascertain the nitrogen content per wet weight of the sample the moisture content of the sample must be known.

Determination of nitrogen in wet tissues fixed in sulfuric acid. The content of the pyknometer with the sample is carefully poured into a Kjeldahl flask. The pyknometer is rinsed with several small portions of ammonia-free water with 1-2 drops of indicator. All washings are combined and poured into the Kjeldahl flask. Rinsing is continued until the washings are not violet. The total volume of washings should not exceed 10 ml. The content of the Kjeldahl flask is mixed and the flask placed on a burner. If the acid is not well mixed with the sample, the sample may explode during heating and the sample may be lost by splashing. Further procedure is exactly the same as in the case of dry samples.

Wet samples fixed in sulfuric acid have a certain advantage over dry samples, namely that they can be analyzed without preliminary drying. On the other hand, the weight of dry samples is not restricted (there is no limit as to the size of the sample). This is of importance in cases when analyses for moisture, ash, fat etc. must also be carried out.

Nitrogen content of the excreta is determined in the same way as the nitrogen content of the fish body. The excreta are dried to constant weight and analyzed in duplicates. The weight of the samples depends on the availability of excreta, in any case the weight must not be below 15-20 mg. If the total amount of excreta is smaller than this, one must dispense with duplicates. For the digestion of such samples, 3 ml of concentrated sulfuric acid are enough. Since excreta contain little nitrogen, the digested sample is diluted in a 25-50 ml volumetric flask. For the distillation 5-10 ml aliquots are taken, depending on the theoretical nitrogen content. At first the percentage

of nitrogen in the sample is calculated. Since the total dry weight of the excreta is known, their total nitrogen content can be calculated. The value obtained is divided by the total weight of all fish studied and the amount of nitrogen excreted during the experiment per 1 g body weight of the fish may be calculated. In this way the amount of nitrogen excreted in the feces of the fish during various times of the day is calculated. As a result a picture of changes in the rate of nitrogen excretion during various parts of the day is obtained. On the basis of these figures the total amount of nitrogen excreted per 1 g of body weight per day or per one fish per day is calculated. If the weight of the excreta is less than 10 mg the digested sample should be distilled without dilution. For this purpose the content of the Kjeldahl flask is quantitatively transferred into the distillation flask. The Kjeldahl flask is rinsed with several portions of ammonia-free water which is also transferred into the distillation flask.

The nitrogen content in water samples withdrawn from each aquarium is also determined in duplicates. Digestion may be carried out in 250 or 100 ml Kjeldahl flasks. In the former case the flask is filled with 100 ml of water to which 2 ml of concentrated sulfuric acid and several crystals of  $\text{CuSO}_4$  are added. The content of the flask must be mixed carefully before boiling. After the water is boiled off, charring occurs. The flask is stoppered to prevent evaporation of sulfuric acid and splashing. Otherwise the entire sulfuric acid may evaporate and the sample is lost.

When digestion is carried out in 100 ml Kjeldahl flasks, 100 ml of water and 1 ml of sulfuric acid are used. Water is added in two portions. At first 50 ml of water are added, and when the water is boiled off another 50 ml portion is added. It is undesirable to add 100 ml of water at once, since it may splash during boiling. Water must be measured with 50 ml pipets. The sample is considered digested when on cooling it remains colorless, transparent or weakly green (due to the presence of  $\text{CuSO}_4$ ). The digested sample is quantitatively transferred into the micro-Kjeldahl apparatus. The Kjeldahl flask is rinsed with several portions of ammonia-free distilled water with several drops of indicator. All washings are combined and poured into the micro-Kjeldahl apparatus. The flask should be rinsed until the water ceases to be violet colored. In all cases rinsing should be done with a similar amount of water.

To ascertain the amount of 33% solution of NaOH which should be taken, a specially designed experiment should be carried out. To a 100 ml conical flask containing sulfuric acid in amounts required for digestion of the sample, and several drops of the indicator, 33% solution of alkali is added in small portions. During the addition of alkali the content of the flask must be mixed. The end-point is indicated by the appearance of a stable green color. Having determined in this way the amount of NaOH needed for the neutralization of the sulfuric acid used for the digestion of the sample, one should use 25% excess of alkali in the experiment proper.

Five ml of 0.01 N sulfuric acid are usually sufficient to trap the entire amount of ammonia distilled off. The condenser tip, especially at the beginning of distillation, should be completely immersed in the sulfuric acid in the receiver, otherwise part of the ammonia can remain unbound and be lost.

If the results of titration of two parallel samples differ by more than 0.05 ml, further samples should be digested and steam distilled. This should be repeated until the results obtained will concur by less than 0.05 ml. The amount of nitrogen excreted by fish is calculated from



the difference in nitrogen content of water from the aquarium containing fish and those without fish. Once the amount of nitrogen excreted by fish in the sample analyzed is known, the total amount of nitrogen excreted by the fish can be calculated by multiplying the value obtained per total volume of water in the aquarium. If the total amount of nitrogen excreted by fish and the weight of all experimental fish are known it is easy to calculate the amount of nitrogen excreted per 1 g of body weight of the fish. The average value obtained from two parallel experiments carried out simultaneously shows the rate of nitrogen excretion during the given time of day. Similar data from other experiments carried out during the same day will show the daily rhythm of nitrogen excretion by fish. On the basis of these data it is easy to calculate the average amount of nitrogen excreted daily per fish of average weight (or per weight unit of fish) per day.

#### CALCULATION OF NITROGEN BALANCE AND FOOD RATIONS

If the average weight of fish and their percent content of nitrogen at the beginning and at end of the experiment are known, increase in nitrogen content in the fish body during this time can be calculated. The difference between the nitrogen content at the beginning and end of the experiment shows the increase in nitrogen in the course of the experiment. This value divided by the number of days elapsed from the beginning to the end of the experiment gives the average increment of nitrogen in the fish body per day. Such a calculation is possible in experiments of short duration and carried out under constant environmental conditions. Nitrogen products of protein metabolism is expressed as an average value of daily nitrogen excretion per fish at the beginning and end of the experiment. In the same way the amount of nitrogen excreted with feces (non-utilized nitrogen) is determined.

Hence the three values (amount of nitrogen deposited in the fish body, amount of nitrogen eliminated with the products of protein metabolism and excreted in the feces) comprise the nitrogen balance sheet. We speak of a positive nitrogen balance when the amount of nitrogen deposited in the fish body exceeds the amount excreted, i.e. protein synthesis exceeds its decomposition. Such a state is characteristic for fish in the stage of growth. Here the balance index reflects the magnitude of nitrogen taken in with food.

The balance may also be negative, when the nitrogen expenditure exceeds the amount of nitrogen taken in. This indicates nitrogen starvation, and if the latter lasts for a long time it may result in death of the organism. A negative nitrogen balance is observed in fish in the winter, when they cease to take food.

For the sake of comparison nitrogen consumption is expressed in percent of body nitrogen. This figure reaches maximum values in fish at early stages of their development, it diminishes with the age of the fish and the increase in size of the fish.

To show what part of the nitrogen taken in with the food is used up for growth, the amount of nitrogen deposited in the fish body is expressed in percent of nitrogen taken in with the food. This figure is called the productive protein effect. It shows the extent to which the organism's requirement for

protein nitrogen is satisfied. Nitrogen in the excreta is expressed in percent of the nitrogen ration, and characterizes the unutilized part of food nitrogen.

In order to be able to proceed from nitrogen consumption to the weight of food eaten, the percent content of nitrogen in the given food must be known. If the experimental fish is kept on a uniform diet this calculation is a relatively simple one and can be made according to the following formula:

$$X = \frac{A \times 100}{B}$$

where X - daily food ration in mg.

A - daily amount of nitrogen consumption, in mg.

B - nitrogen content in food, percent per wet weight of food

We shall illustrate this with an example. Let us assume that a fish fed on bloodworm which contain 1.55% nitrogen per wet weight has consumed 4.5 mg of nitrogen per day. The daily ration is then equal to  $\frac{4.5 \times 100}{1.55} =$

290.3 mg wet weight of food.

For the sake of comparison, the food ration and nitrogen ration are expressed in percent of the average body weight of the fish. If fish are kept on a mixed diet (as is the case under natural conditions) the calculation is somewhat complicated. In this case both the amount of food (in wet weight) and its nitrogen content must be known. To this end the intestinal contents of 10-20 fish (depending on the food variety) is analyzed at the beginning and end of the experiment. The average food content (average number of organisms eaten) and its nitrogen content during the entire duration of the experiment are calculated. When large fish are studied it is not necessary to analyze the entire content of fish intestines. Weighed samples may be analyzed - these may be taken from different parts of the intestine or as aliquots from the total intestinal content. It is better to take the content of the anterior segment of the intestines, where the food is still undigested.

When the average number of different species eaten by the fish, and their nitrogen percent content are determined, their total wet weight and then their total nitrogen content are calculated. On the basis of these data the nitrogen content of various organisms eaten can be determined. This allows for the determination of the fraction of nitrogen utilized from this or another species, and for calculation of the weight of each food. This calculation is carried out according to the formula given above, with the only difference that value A (daily nitrogen consumption) is substituted by the amount of nitrogen consumed with each of the different foods. The sum of all values calculated in this way indicates the daily food ration. In Table I an example of calculation of the food ration for sazan fry weighing 6.0 g and consuming 17.9 mg of nitrogen daily is presented.

Analysis of intestinal contents showed that sazan feeds on bloodworm larvae and mollusks. According to the reconstructed weights, the intestines analyzed contained 400 mg of larvae and 400 mg of mollusks on the average. Assuming that bloodworm larvae contain 1.55% of nitrogen per wet weight, while mollusks contain 0.67% nitrogen, it may be seen that 400 mg of bloodworm contain 6.20 mg nitrogen, and 400 mg of mollusks - 2.68 mg of nitrogen

Bloodworm comprise 69.8%, and mollusks 30.2% of the total nitrogen content of fish intestines. On the basis of these figures, we calculate that from the total nitrogen ration equal to 17.9 mg, bloodworm larvae provide 12.49 mg, and mollusks 5.41 mg. Since bloodworm larvae contain 1.55% of nitrogen, the 12.49 mg of nitrogen indicated correspond to 806 mg of wet weight, and 5.41 mg of mollusks containing 0.67% of nitrogen correspond to 807 mg of wet weight. Hence the total food ration comprises 1613 mg, or 26.9% of body weight.

TABLE 1  
Calculation of food rations for sazan fry

Index	Food		
	bloodworm larvae	mollusks	total
Weight of intestinal content, in mg . . . . .	400	400	800
Nitrogen content, percent of wet weight . . . .	1.55	0.67	-
Food nitrogen content, in mg . . . . .	6.20	2.68	8.88
Percent nitrogen content in the various foods .	69.8	30.2	100.0
Daily consumption of nitrogen, in mg . . . . .	12.49	5.41	17.90
Consumed per day per wet weight, in mg . . .	806	807	1613

TABLE 2  
The content of moisture and nitrogen in the food items of fish

Organisms	Moisture, %	Nitrogen content, %	
		per wet weight	per dry weight
Lower algae	90.08	0.46	4.68
Higher plants	84.80	0.38	2.46
Bryozoa (moss animalcules)	85.45	0.67	4.56
Rotifers (wheel animalcules)	-	-	8.75
Copepods	87.50	1.13	9.12
Cladocerans (waterfleas)	87.40	0.99	9.28
Aphipoda (sand hoppers)	80.39	1.57	7.99
Phyllipoda (fairy shrimp)	89.60	0.72	6.90
Isopods (sowbugs)	78.24	2.47	11.37
Sewage worms	83.75	1.36	8.39
Oligochaetous worms (tubificid worms)	82.07	1.70	9.51
Polychaetous worms (sandworms, tube worms)	72.37	2.62	9.54
Mollusks	70.85	0.67	2.27
Bloodworm (larvae)	83.72	1.55	9.45
Dragonfly larvae	80.08	2.19	11.00
Caddis fly larvae	77.45	1.81	8.80
Ephemeridae larvae	80.46	1.82	9.32
Stonefly larvae	83.44	1.97	11.91
Beetle larvae	82.80	1.58	9.18
Bugs	78.50	2.07	9.65
Frog tadpoles	92.40	0.67	8.84

From this example the nutritional value of various foods is clearly seen. On the same weight basis, butterfly larvae have a  $2\frac{1}{2}$  times as high nitrogen content.

To determine the average wet weights of food components (organisms eaten by the fish) they are weighed after being blotted on a filter paper. In addition, the data on wet weights of aquatic animals which serve as food for the fish are given in numerous hydrobiological works.

The nitrogen content of various foods varies depending on the age, locality, and season. For accurate calculations, the nitrogen content of various food objects from the reservoir from which the fish are studied should be determined. Determination of nitrogen content of all food objects is unnecessary; 3-4 determinations on each representative of the order or class of food objects are sufficient. An average of the data which characterize nitrogen content of various organisms may then be made.

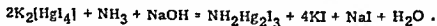
Data from literature and our own research on nitrogen content in some main freshwater organisms are given in Table II.

## METHODS FOR AMMONIA DETERMINATION USED IN STUDIES ON FISH METABOLISM

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Ammonia, urea, uric acid and other substances constitute the final products of protein metabolism in fish. The bulk is comprised of ammonia and urea. Nitrogen excreted in the form of ammonia amounts to 85% of all nitrogenous substances excreted. Hence, in order to be able to assess the rate of protein metabolism, the amount of ammonia excreted must be known. The most reliable and accurate method for ammonia determination is the colorimetric method of Nessler. Nessler reagent consists of a colorless alkali solution of the complex salt of  $K_2HgI_4$  which reacts with ammonia and ammonium ions, with the formation of yellow mercurammonium. The intensity of the color produced is proportional to the ammonia concentration. The reaction proceeds according to the following formula:



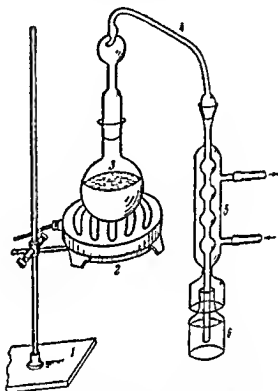
Other salts such as  $CaCO_3$ ,  $MgCO_3$ ,  $Mg(HCl)_2$ ,  $(HCO_3)_2$ ,  $Ca(HCO_3)_2$  may interfere with the reaction. To avoid interference from the above salts, ammonia should be distilled prior to nesslerization.

Distillation of ammonia from the sample. The distillation apparatus is depicted in the Figure.

At first the apparatus must be freed of  $CO_2$ . For this purpose the 300-350 ml distillation flask is filled with 150-175 ml of water. Tap water is preferred, because the ammonia content of the ordinary distilled water may be quite high. To the tap water a small amount of  $MgO$  is added on the end of knife or spatula, which renders the water slightly alkaline. The flask is placed on an electric plate or alcohol lamp and tested. At the same time water is circulated through the condenser and a pyknometer-receiver is placed underneath. Due to the alkaline pH of water (in the presence of  $MgO$  the pH of the water at the end of distillation rises to 8.5-9.0) ammonium salts are liberated and distill off with the water vapor, condense in the condenser and are collected in the receiver.

The first portions of the distillate contain large amounts of ammonia, and the amount of ammonia in the distillate rapidly decreases, so that the total ammonia content of the flask distills over rapidly. It is advisable to follow the changes in ammonia concentration in the distillate in the first

few experiments to determine how rapidly ammonia distills off. According to our observations, ammonia can be transferred quantitatively into the receiver by distilling off 2/3 of the initial volume of the water. At the end of distillation a certain amount of water, approximately 30-40 ml, with undissolved MgO should remain in the distillation flask. This water does not have to be poured out since it does not contain ammonia, and the apparatus may now be used for the determination of ammonia in the unknown sample.



Apparatus for ammonia distillation from the sample

1—prop; 2—electric heater; 3—distillation flask; 4—trap;  
5—spherical condenser 6—receiver.

When the apparatus has cooled down, the trap (4) is carefully removed, the flask is placed in a rubber ring, and 100 ml of the sample are poured into the flask by means of a volumetric Mohr pipet. Water should be poured out slowly and vigorous stirring should be avoided. To this end the pointed tip of the pipet is placed on the bottom of the flask below the surface of water present in the flask and the sample is allowed to flow slowly into the flask. With the decrease in the amount of water in the pipet, the sample is allowed to flow more rapidly by lifting the finger from the upper end of the pipet. The last drops of the sample are removed from the pipet in the following way, the upper end of the pipet is closed with the index finger of the right hand, while the lower end of the pipet touches the inner edge of the

neck of the flask, and the left hand holds the bulb of the pipet. From the warmth of the hand holding the distended part of the pipet, the air in it expands and displaces the last water drops from the pipet. No  $\text{MgO}$  need be added. The flask is placed on the electric heater and the trap connecting the distillation flask with the condenser is put in place. The trap is joined to the distillation flask and the condenser by means of ground glass joints. Simultaneously, the receiver which is rinsed with ammonia free water is placed under the condenser. The pyknometer is placed in such a manner that the condenser tip almost touches the bottom. The pyknometer should have a mark (inked or pencilled in) corresponding to 100 ml volume. When everything is ready, the electric heater is switched on, water is allowed to circulate through the condenser, and the sample is distilled.

With the accumulation of distillate in the receiver the latter is lowered so that the tip of the condenser barely touches the distillate. When the amount of the distillate in the receiver reaches the 100 ml mark, the receiver is removed and stoppered. The heater is switched off. The distillate contains ammonia dissolved in water which has distilled over. The concentration of ammonia in the distillate (in 100 ml of the distillate) is the same as it was in the sample (in 100 ml of the sample).

Ammonia distillation in such an apparatus meets with no difficulties and yields good results, provided one adheres to the instructions given above. It must be remembered that the air of the laboratory should be free of ammonia, smoke and other contaminants. Pure air of the room, under otherwise similar conditions, ensures the reliability of the results obtained. Tobacco smoke, for example, in conjunction with Nessler's reagent, renders distilled water greenish, which may interfere with the colorimetric method of ammonia determination.

**Colorimetry** The distillate (weak ammonia solution) may be stored in the pyknometer for 1-5 days. Storage for 1-5 days does not lead to any changes in ammonia concentrations. Since the air in the laboratory often contains ammonia, care must be taken that the latter should not find its way to the pyknometer with the distillate during the colorimetric determination. Tobacco smoke should be avoided in particular.

For serial routine colorimetric ammonia determinations it is better not to use standard solutions of ammonium chloride, but an inorganic artificial standard consisting of a composition of  $\text{K}_2\text{PtCl}_6$  and  $\text{CoCl}_2$  solutions according to a certain color range. For this purpose the following solutions must be prepared.  $\text{CoCl}_2$  (2 g of the salt is dissolved in a small amount of water. 100 ml of concentrated  $\text{HCl}$  are then added and the volume made up to 1,000 ml),  $\text{K}_2\text{PtCl}_6$  (12 g of this salt + 100 ml of concentrated  $\text{HCl}$  diluted to 1,000 ml),  $\text{NH}_4\text{Cl}$  containing 0.25 g  $\text{N}/1,000$  ml and Nessler reagent. The composition of the Nessler reagent may differ. Any reagent may be used, and one must choose the one available for this determination. Several examples of a preparation of Nessler's reagent from different chemicals are presented in Table 1.

It can be seen from Table 1 that various investigators took not only different ratios of these substances, but actually used different initial chemicals. Although an alkaline solution of  $\text{K}_2\text{HgI}_4$  is obtained, the method of preparation is not without effect on the quality of the reagent.

The following method may be used for the preparation of Nessler reagent. For the dissolving of the salts 100 ml of water are prepared. Then 10 g of  $\text{HgI}_2$  are carefully ground in a porcelain mortar with a small amount

of water. This slurry is rinsed with water and poured into a beaker to which 5 g of KI dissolved in a small amount of water are added, 20 g of NaOH are dissolved in 50-60 ml of water, and after the solution has cooled down it is poured into the beaker. The remaining amount of water is added into the same beaker, 10-15 days later the solution becomes transparent and colorless, and is suitable for ammonia determinations. The reagent should be stored in a dark bottle in a dark place (ground stoppers are not advisable).

TABLE 1

Ratio of components comprising Nessler reagent, according to various authors

Author of the prescription	Initial salts in g								Water
	KI	HgI <sub>2</sub>	HgCl <sub>2</sub>	I <sub>2</sub>	KBr	Hg	NaOH	KOH	
Winkler	25	10	—	—	50	—	25	—	T
Winkler	50	100	—	—	—	—	200	—	D
Folin	30	—	—	22.5	—	30	100	—	D
Bruevich	124	—	60	—	—	—	—	150	D
Jou	25	35	—	—	—	—	—	150	D
Malinina	50	—	30 + 5 ml of saturated solution	—	—	—	—	—	D
Laboratory methods for U.S.A. army	50	—	Saturated solution to the end of the reaction	—	—	—	—	144	D
"Sputnik khimika" (Chemist's Guide)	50	70	—	—	—	—	—	150	D
Aleida	80	115	—	—	—	—	120	—	D

Remark All the data are calculated per 1,000 ml of the reagent D—distilled water; T—tap water

For colorimetric determinations 26 60ml pyknometers (beakers) from pure white glass and of identical diameter should be used. Pyknometers are chosen according to their color. Each pyknometer is then filled with 50 ml of water, accurately added with the aid of a volumetric pipet. Pyknometers with identical diameters are selected (This is judged by the water level.) This level is marked by India ink or paint (a ring mark is made). Then the pyknometers are enumerated from 0 to 12 in duplicates, i.e., two similar rows of pyknometers are obtained.

Pyknometers of the first row are filled with the following solutions, and in the ratios given in Table 2, the volume of each pyknometer is then made up to 50 ml (to the mark) with distilled water.

Pyknometer 0 is filled with distilled water (contains no ammonia with Nessler reagent). In the remaining pyknometers the number of pyknometer also indicates the concentration of nitrogen of ammonia (0.1 mg N/1000 ml, 0.2 mg N/1000 ml, . . . . . 1.2 mg/1000 ml).

Before this scale is used, it should be matched with a standard solution of NH<sub>4</sub>Cl. For this purpose a standard solution of recrystallized NH<sub>4</sub>Cl is prepared. Ammonium chloride used should be dried at 100°C. 0.0382 g



of this salt are dissolved in 1,000 ml of ammonia-free distilled water. 1 ml of such a solution contains 0.01 mg N. 1 ml of this solution is added to a 100 ml volumetric flask, and the volume is made up with ammonia-free distilled water. 50 ml of aliquot are poured into pyknometer No. 1 of the second row, 1 ml of Nessler reagent is added, the pyknometer stoppered, and the contents are mixed.

TABLE 2  
Solutions for preparation of Nessler reagent

Pyknometer	Solution, ml		Concentration
	CoCl <sub>2</sub>	K <sub>2</sub> PtCl <sub>6</sub>	
0	0	0	0
1	0.11	4.76	0.1
2	0.50	7.70	0.2
3	1.12	10.00	0.3
4	2.20	12.70	0.4
5	3.30	15.00	0.5
6	4.50	17.30	0.6
7	5.70	19.00	0.7
8	7.10	12.70	0.8
9	8.70	19.90	0.9
10	10.40	20.00	1.0
11	12.50	20.00	1.1
12	15.00	20.00	1.2

In this way NH<sub>4</sub>Cl solutions for pyknometers 2.....12 of the second row are prepared. 2-12 ml of the initial NH<sub>4</sub>Cl solution are added to 100 ml volumetric flasks and their volume is made up with ammonia-free distilled water. 50 ml aliquots from the volumetric flasks are poured into the corresponding pyknometers. Hence the pyknometers of the second row contain 0.1, 0.2, 0.3, .....1.2 mg N/1,000 ml.

Maximum color intensity is reached after 5-10 minutes. After this time the pyknometers of the second row are compared with analogous pyknometers of the first row (i.e., inorganic scale). If this scale differs somewhat in color from that of pyknometers of the second row, the latter can be brought to the same color intensity by adding CoCl<sub>2</sub> or K<sub>2</sub>PtCl<sub>6</sub> solutions. In this way each pyknometer of the (mineral) inorganic scale is treated (always matched with the color of the pyknometer with the corresponding concentration of NH<sub>4</sub>Cl). Having verified the inorganic scale, the pyknometers should be stoppered and sealed (by means of wax, paraffin, putty, asphalt, etc.).

The scale prepared in this way may be used for several years. After verification the solutions from the pyknometers of the second row are discarded. Two pyknometers are marked by x<sub>1</sub>—first sample, and x<sub>2</sub>—second sample. The remaining pyknometers remain in reserve.

Ammonia in the distillate is determined in the following manner. Pyknometer x<sub>1</sub> is rinsed with a small amount of the distillate. The pyknometer is filled with the distillate to the mark (50 ml) and 1 ml of Nessler reagent

is added. The pyknometer is stoppered and gently mixed. After 10 minutes of standing, the sample  $x_1$  is matched with one of the standard pyknometers

Ammonia concentration in sample  $x_1$  is determined by comparing and interpolating with the inorganic scale. Since our eye can better discern the difference in color intensity by contrast, the sample should be placed between two pyknometers with ammonia concentration higher and lower than that of the sample. By interpolation, one hundredth of a mg of N/1,000 ml can be determined. The interpolation involves division by two or three. The inorganic scale may be calibrated into smaller divisions (interval of 0.05 mg N/1,000 ml). In this case the determination is easier and the color of the sample  $x_1$  can be matched more easily with that of the scale.

Comparison is made easier by placing white filter paper under the pyknometers. Care should be taken that no shadow or beam of light will fall on the pyknometers (this may interfere with accurate determination).

The method described above allows one to determine ammonia with an accuracy of 0.01 mg N/1,000 ml almost simultaneously in two samples ( $x_1$  and  $x_2$ ).

Colorimetric determinations of ammonia with the aid of Nessler reagent carried out in Dubosque colorimeters offer no advantage over our method (neither in speed nor in accuracy). In many instances it gives less accurate results than the method described above. It should be remembered that Nessler reagent gives a color reaction not only with ammonia ions but also with amines. Only complex mercuric iodide gives a strong color with ammonia. Other complexes, such as  $\text{Na}_2\text{HgCl}_4$ , are colorless and poorly soluble compounds. Some chemists believe that in the presence of other salts the iodide complexes may undergo exchange reactions which come to equilibria with such colorless complexes as chlorides and sulfates.

Maximum color intensity is reached after a certain time, which depends on the method by which the Nessler reagent had been prepared, and the amount of alkali and the ratio between the volume of the sample and that of the Nessler reagent. The more alkali in the reagent and the more reagent is added to the sample, the less time is needed for maximal development of the color. A high concentration of alkali in the Nessler reagent, however, causes a slight turbidity in the sample, even in the absence of alkali earth metals which interfere with colorimetric determinations.

From the above-said it is clear that for our purpose the latter must be distilled off prior to ammonia determination, as described above.

The air in the laboratory must be clean and ammonia-free. During distillation the air should be kept free of fumes and especially of tobacco smoke. The amount of ammonia nitrogen excreted by the fish can be calculated from the following formula:

$$\frac{(m_1 - m_0)V}{pt} = N, \text{ mg per 1 g body weight per one hour,}$$

where  $m_0$  and  $m_1$  — ammonia concentration before and after the experiment, expressed in mg N/1,000 ml water.  
 $V$  — volume of water (in liters) in which the fish has respired,  
 $p$  — weight of fish, in g.  
 $t$  — duration of experiment, in hours

## DETERMINATION OF FAT IN THE FISH BODY

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### COLLECTION OF DATA

The fat content in fish varies, depending on numerous factors - life conditions, season of the year, nature and intensity of nutrition, age, sex, the stage of ripeness of sexual products, the number of developing eggs, etc. Hence, the object of study of the fat content in fish should be collected according to the aim of the investigations. In all cases, the fish should be differentiated according to their age and size, their sex and maturity of their sexual products. It is not enough to take the results of analysis from fish of different size and stage of maturity in order that the collected material reflect the true changes occurring in the fish organism. One must consider the following factors: 1) the place from where the sample is taken; 2) time (season), 3) the depth and temperature of the water; 4) the state of nutrition, 5) the length of the fish and, if possible, its age; 6) sex, 7) the stage of ripeness of the sexual products.

Since the fat content in fish is subject to wide individual variations, more accurate data can be obtained when pooled samples of several fish of the same size, age, sex and degree of maturity of sexual products are investigated. The number of fish to be taken for one investigation depends on the type of fish and numbers available. An average sample of small fish (herrings, sprats or anchovy) should contain not fewer than 10-20 specimens.

Fish of approximately the same size should be chosen in the experiments. If the sample consists of specimens which differ markedly from each other in length, small and large fish should be analyzed separately, otherwise the results obtained will not be representative. It is also inadvisable to mix fish of different sex and with sexual products in various stages of maturity.

If the studies are aimed at determinations of the effect of fat content in fish, and when the age of the fish in question cannot be determined, specimens should be taken which in size approach each age group. Sexes should not be mixed, and the stage of sexual maturity must be considered.

In order to investigate the changes in fat content of fish during sexual maturity, fish of similar size are chosen and divided into groups according to their sex and stages of maturity.

Depending on the aim of the study and the fish studied, whole fish or some body parts are analyzed. Small fish are usually taken in toto. From large fish separate organs may be analyzed. If the fat content of muscles is to be analyzed, muscles from one and the same body part must be taken.

Fat distribution according to body parts varies, depending on the fish species. For example, in herrings and sturgeons the bulk of fat is concentrated in the muscles, in cods, fat is concentrated in the liver, while in perch fat is concentrated in the body cavity. This must be considered when analysis of various parts of the fish is made. If one cannot analyze all organs, the characteristic features of the fat content in the fish in question may be ascertained by analyzing the fat content of organs in which the bulk of fat is concentrated. Thus, for example, data on the fat content of lean body parts, as in cod muscles, will not be representative of the contents in the fish. The fat content in the body of the fish changes depending on environmental conditions, or on changes taking place in the fish organism (for example, during asexual maturation), and on the state of nutrition, etc. In summer, these changes are more rapid than in winter. For studies on seasonal fat contents, the material should be collected in the summer every fortnight, and in winter every 30 days. When marked changes in the physiological state of the fish, as in the spawning, are noted, the next sample should be taken immediately after this state is detected, irrespective of the date of the preceding sample.

Samples of fish or parts of the fish body are minced and homogenized. The homogenate is weighed and the weight of the sample recorded. The sample may be stored in a glass weighing bottle after drying it to an air-dry state in a thermostat at 70-80°C, and stored in a cool place. The samples can also be stored in tins which should be sealed, wrapped and autoclaved. Samples may also be stored in sealed test tubes. They may also be closed with a baby's soother and tied with a thread. The test tubes are then sterilized in boiling water for two hours and stored in a cool place.

#### QUANTITATIVE DETERMINATION OF FAT

There are numerous methods of fat determination. The most widespread method is that based on weighing the defatted residue (Rushkovskii's method). This method is convenient in that it permits several analyses to be performed simultaneously. This method requires that the sample be weighed and enclosed in two envelopes of filter paper and placed in a Soxhlet apparatus for fat extraction. After extraction the sample is reweighed. Fat content is determined from the difference in the weight of the sample before and after extraction.

The sample to be analyzed is placed in a glass weighing bottle and dried in a thermostat at 65-70°C to constant weight. Moisture content is determined from the difference in weight of the wet and dry sample, and is expressed in percent of wet weight. The dry sample is carefully ground in a porcelain mortar. Since in the course of grinding the sample takes up moisture, it must again be dried to constant weight in a thermostat at 60-70°C. Then two envelopes are made from filter paper. One envelope is inserted into the other. The inner envelope is prepared from a 6x7 cm filter paper, and the outer envelope from a 7x8 cm filter paper.

The envelopes are made in the following way: a filter paper sheet is folded so that one side protrudes over the other, forming a margin 4-5 mm wide. This margin is then folded twice. One end of the envelope is left free (through this end the sample is poured in), while the other end is

sealed (to prevent the sample from spilling out) For this purpose the end of the envelope is folded together with the margin along the entire length of the packet at an angle of  $90^\circ$ , and the triangle formed is folded in two by placing its tip under the margin. In the same way the second end of the envelope is closed after placing the sample in it.

The packets, closed from one end and numbered (the numbers are pencilled in) are defatted with ether in the soxhlet apparatus for 4-5 hours. After the extraction, the small and large envelopes are placed in one weighing bottle and dried to constant weight in a thermostat. Samples weighing 100-500 mg are then placed in the inner envelope, and the envelope is closed and inserted into the outer one which is also closed. The outer envelope serves for preventing any losses of the sample during extraction in the soxhlet apparatus.

The weighed portion is determined by weight difference. First, the weighing bottle with the sample is weighed on an analytical balance and the desired amount of the sample is then transferred by means of scalpel or fine spatula into the envelope. The weighing bottle is then reweighed. The difference between the initial weight and that after the sample withdrawal represents the weight of the sample.

A series of envelopes with samples are placed in a soxhlet apparatus and the samples are extracted with petroleum ether or sulfate ether for 10-15 hours. The duration of extraction depends on the number of samples and their fat content. After extraction, the envelopes were reopened, placed in the corresponding weighing bottles, and dried to constant weight in a thermostat. The difference between the weight of the sample before and after extraction (defatted residue) represents the weight of fat in the sample. The weight of fat is expressed in percent of dry weight. If the moisture content (percentage) of the sample is known, the fat content may be expressed in percent of wet weight.

To obtain a greater accuracy and to prevent possible errors the analysis is carried out in duplicates. The soxhlet apparatus for fat extraction (Figure 1) consists of an extractor 1, which is connected from below, by means of ground glass joints, with flask 2 and from above with a reflux condenser (through which tap water circulates). The flask is filled to  $3/4$  of its capacity with dry petroleum ether or sulfate ether, and heated on an electric heater (sand bath or water bath may also be used). The ether should not boil during heating (ether boils at  $40^\circ\text{C}$ ). The flask should be heated gently since the ether is easily inflammable. During heating ether vapors rise along the side tube 3 and condense in the condenser. Condensed ether accumulates in the extractor and dissolves the fat of the sample. After the height of the ether column in the extractor reaches the upper part of the siphon 4 it returns back to the flask, again evaporates, and the entire process is repeated until the entire fat content is dissolved and transferred into the flask.

In spite of the high accuracy of this method and the possibility to test several samples simultaneously, it is not the best method since it is time-consuming (time needed for extraction and drying to constant weight). Generally, analysis of one series of samples takes 4-5 days. For this reason numerous investigators looked for other more rapid methods of fat determination.

In the Laboratory of Physiology of VNIRO the modified method of V. I. Bel'kevich, originally developed for fat determination in insects was applied. The method requires that the sample be first dehydrated by grinding with ignited sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) or disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ), and then be treated with a given amount of dichloroethane in a closed test tube in a water bath at  $37-40^\circ\text{C}$  for 30 minutes. At the end of extraction an aliquot is transferred into a weighing bottle of known weight. The aliquot should be transferred into the weighing bottle by means of a graduated pipet. Dichloroethane is distilled off in a special apparatus and the weighing bottle reweighed. The difference between the weight of the weighing bottle before and after distillation shows the weight of fat dissolved in dichloroethane. Knowing the weight of fat in the solvent, the total fat content of the sample can be easily determined.

For analysis 2 g samples (in duplicates) of wet weight of homogenate are carefully ground in a porcelain mortar with three times its weight of ignited  $\text{Na}_2\text{SO}_4$  (sodium sulfate) or disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 5\text{H}_2\text{O}$ ). The sample is ground to a dry powder. Grinding also facilitates cell disruption which promotes a rapid and quantitative fat extraction.

The ground sample is placed in a thick wall round bottom test tube 20 cm high and 3.0 cm in diameter. Sodium sulfate and particles of the sample which adhere to the mortar are removed with a spatula and again ground with an additional 0.5 g of sodium sulfate. The entire sample is then collected in the test tube. This procedure is repeated twice so that the fat is quantitatively removed from the mortar and pestle and absorbed by sodium sulfate. The sample should be ground very carefully to avoid losses. There is no danger of adding sodium sulfate in excess, since the latter does not interfere with the determination. To make this method more rapid, sodium sulfate may be taken without weighing (in spoons or other measuring devices).

The ground sample is placed in a test tube to which 20 ml of pure dichloroethane are added. Dichloroethane is a poison and should be pipetted with a Knudsen automatic pipet with a two way stopcock. The rubber stopper of the bottle in which dichloroethane is stored should have two holes. Through one hole the Knudsen pipet is inserted so that its tip is immersed in dichloroethane. Through the other hole passes a bent glass tube connected with a rubber bulb or a bicycle pump. By pumping air into the bottle dichloroethane is forced into the pipet.

After dichloroethane is poured into the test tube its content should be stirred by means of glass pestle (to improve extraction). Care should be taken that during this procedure dichloroethane does not evaporate. For this purpose the test tube is closed with a baby's soother provided with a hole through which the pestle passes. The opening of the soother should fit the pestle tightly (Figure 2, A). As a result, a mobile joint is formed which permits stirring and precludes losses of dichloroethane by evaporation. Since dichloroethane dissolves rubber, care should be taken that during the stirring dichloroethane will not reach the rubber soother.

The test tube is closed. In this manner it is placed in a water bath at  $37^\circ\text{C}$  for 30-50 minutes and occasionally stirred. After this time interval the test tube is cooled to room temperature in a stream of tap water. When the temperature of the test tube has reached that of the room the soother is

removed together with the pestle, and the test tube is stoppered with a rubber stopper with two holes. Through one hole a glass tube 25 cm long is inserted, its lower tip should be 1 cm above the bottom of the test tube. The lower part of the tube (approximately 1/4 of its length) is tightly packed with pieces of gauze previously defatted by extraction with ether. Cotton wool must not be used since it becomes rapidly clogged. Through the upper tip of the tubular filter a 10 ml Knudsen pipet is inserted until its tip touches the gauze packed in the lower part of the tube. To prevent passage of air between the walls of the tube and the pipet, they are connected by means of a short rubber tubing (Figure 2, B). The second hole serves for inserting a glass tube connected with a rubber bulb or a bicycle pump (Figure 2, C). When air is pumped into the test tube, dichloroethane is forced through the gauze and into the pipet. Care should be taken that dichloroethane does not contain particles of the sample. When the pipet is filled with the extract, it should be carefully disconnected from the glass tube and the solution transferred into a weighing bottle of known weight.

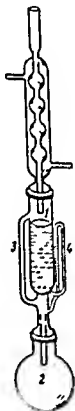


FIGURE 1 Soxhlet apparatus  
1-extractor 2-flask  
3-side tube 4-siphon

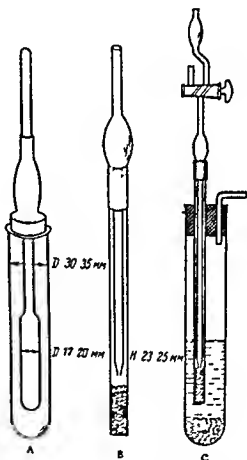


FIGURE 2 Apparatus for fat extraction

A-test tube with a pestle for sample grinding  
B-filter C-test tube during removal of solution by suction.

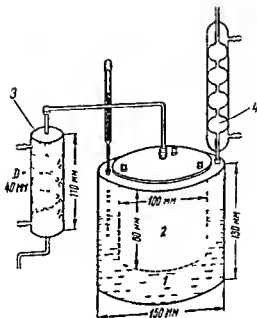


FIGURE 3 Distillation apparatus

1—glycerol-water mixture 2—distillation flask 3—condenser for the distilling solvent 4—reflux condenser for water condensation.

The weighing bottle with the sample is stoppered and placed in a special apparatus for dichloroethane distillation at 125-130°C. This apparatus is in the form of a double-walled copper or brass cylinder (Figure 3). The inner chamber of the distillation apparatus is closed with a lid. To avoid losses of dichloroethane the lid is hermetically screwed. From the center of the lid an inverted L-shaped brass tube passes, and connects the chamber with a brass condenser for cooling dichloroethane vapors. On two sides of the lid there are two openings leading into the interspace filled with glycerol-water mixture. This mixture is prepared so that its boiling point should not exceed 125-130°C. For this purpose 75 parts of glycerol and 25 parts of water are taken. If the boiling point is too high, water may be added; if, on the other hand, the boiling point is too low, some water is removed by evaporation. A thermometer is inserted into one of the openings and a reflux condenser into the other. Water vapor which condenses in the condenser thus flows back, so that the glycerol concentration and boiling point of the mixture remain constant.

Dichloroethane of the sample should distill for 30-40 minutes. When distillation is completed, the lid is removed and the apparatus is left open for several minutes to allow evaporation of the last traces of dichloroethane (as judged by the smell). The glass bottles with the samples are then transferred into a desiccator. When the samples have cooled they are weighed on an analytical balance. The difference between the initial and present weight of the weighing bottle indicates the fat content of one half volume of the



sample. Multiplying the value obtained by two, the fat content in the whole sample can be ascertained.

This method can be used for determination of fat in dry substances. For this purpose 100-500 mg samples of the substance examined are placed in small casseroles or beakers and poured over with 2-3 ml of hot distilled water. The beaker is covered with a watch glass and placed for 10 minutes in a boiling water bath. When the sample is steamed it is poured out into a mortar containing 2-3 g of sodium sulfate. The sample is quantitatively removed from the beaker by repeated wiping of its walls with a small amount of sodium sulfate, which is also placed in the mortar. Further analysis is carried out in the same way as with the wet substances. The sample should be steamed so that the partly moistened sodium sulfate forms a homogenous paste with the sample in the course of crystallization during subsequent treatment with dichloroethane. This procedure facilitates filtration of the fat solution. Otherwise fine particles of the sample and sodium sulfate may penetrate the filter or clog it altogether.

#### APPARATUS AND REAGENTS

For the analysis of fat with our method the following apparatus and reagents are required

- 1) analytical or semianalytical balance,
- 2) meat mincer (homogenizer)
- 3) porcelain mortar,
- 4) thick-walled round-bottom test tubes, 20 cm high and of 28-30 mm inner diameter,
- 5) glass pesties for stirring,
- 6) tubular glass filters,
- 7) 15-25 ml weighing bottles,
- 8) 20 and 10 ml Knudsen pipets with stopcocks,
- 9) apparatus for dichloroethane distillation,
- 10) rubber baby's soothers or rubberfingers,
- 11) rubber stoppers for test tubes,
- 12) bicycle pump,
- 13) defatted gauze,
- 14) anhydrous sodium sulfate ( $\text{Na}_2\text{SO}_4$ ) or disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 5\text{H}_2\text{O}$ ),
- 15) purified dichloroethane,
- 16) glycerol

## METHODS FOR FAT DETERMINATION UNDER FIELD CONDITIONS

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Usually the fat content of fish is determined chemically in the laboratory. Under field conditions, preliminary data on fat content can be obtained from easily determinable indexes such as nutritional state and degree of fatness of internal organs, which can be determined visually and expressed in arbitrary units. These indicators have serious drawbacks. The nutritional state of the animal is usually determined by the Fulton coefficient.

Clark's coefficient of the general state of nutrition is somewhat more convenient.

The essential drawback of both Fulton and Clark's coefficients is that they do not necessarily represent the fat content. These coefficients must therefore be used with caution.

Fatness of the inner organs is usually determined visually and expressed in arbitrary units (4-5 units). As any other visual observation, this method is subjective and the results obtained are often contradictory and cannot be compared.

This chemical method is tedious and calls for appropriately equipped laboratories. For field experiments, however, a relatively simple and at the same time reliable method is required. We recommend to determine the fat content in fish under field conditions by using two criteria—the fatness coefficient and fatness factor of Tester.

Fatness coefficient is the ratio of weight of fat collected from the inner organs to body weight, expressed in percent. This criterion gives a better representation of the extent of fatness of the inner organs than the visual determination.

A very convenient method for determining fatness under field conditions is that of Tester (1941). Unfortunately, it has not been widely used, although its principle has been explained by I. Ya. Kleimenov (1941). The problem of a rapid and accurate method for the determination of fatness (condition) of fish has long aroused the interest of various investigators. The first steps in this direction were made by Bull in 1896. He thought that fatness should reflect the fat content of the organism, according to Bull, fatness (condition) and fat content are synonymous.

The method of Tester is based on the assumption that the specific gravity of the fat tissues is lower than that of lean ones. The specific gravity of

tissues is determined by weighing the organ or organism in air and in water. This is calculated from the formula

$$G = \frac{W_a \cdot K}{W_a - W_w}$$

where  $W_a$ —body weight in the air,

$W_w$ —body weight in water,

$K$ —density of water

Later Tester introduced the term fatness factor

$$F = \frac{G}{G-1} = \frac{W_a}{W_w}$$

Tester's formula in its final form is:

$$F = \frac{W_a}{W_w}$$

The fatness factor (coefficient) has the following advantages over specific gravity: 1) it is easy to determine, 2) changes of  $F$  are more easily detectable than changes in  $G$ ; 3) the value of  $F$  changes according to the fat content. Hence for determination of the fatness factor (coefficient) it is sufficient to divide the weight of the fish in air by its weight in water.

The fatness factor (coefficient) may be determined in a whole fish as well as in its various parts and organs. Fatness factor (coefficient) of a whole fish is determined in the following way: the fish is disemboweled, washed, wiped dry and weighed as accurately as possible; afterwards it is weighed in water, the weight in air is divided by the weight in water. For weighing fish in water Tester used a specially designed balance; we have used somewhat modified pharmaceutical scales. Prior to weighing large fish in water it is advisable to cut the fish just in front of the dorsal fin, to put both halves together, to tie them by means of a hook and to lower them into a bucket filled with water. During immersion the gill covers should be lifted and no air should be left in the abdominal and branchiostegal cavities. In addition, during the weighing the fish should not touch the walls or bottom of the bucket. The fish should always be weighed at one and the same water temperature. The weight of fish in water may change depending on water temperature, due to changes in specific gravity of water at varying temperatures.

As an example of determining the fatness factor in eviscerated fish, we would like to present our data on Atlantic-Scandinavian herrings (Shubnikov, 1959, 1960). The table shows a correlation between the fatness factor (coefficient) of a herring carcass and its fat content in percent of wet weight, the latter was determined by chemical means (extraction in soxhlet apparatus by means of Rushkovskii's method). The correlation coefficient of these two values is + 0.82, i.e., a very high correlation.

By calculating the fatness factor (coefficient) for determination of splanchnic fat and the fatness factor (coefficient) in a whole fish or in eviscerated fish (subcutaneous fat and that of muscle), one is able under field conditions to obtain an estimate of the fat content in the fish group studied (provided a large number of specimens is examined).

TABLE

Correlation between fat content and Tester's fatness factor (coefficient)  
in Atlantic-Scandinavian herrings

Fat content, %	Fatness factor (coefficient)									Total
	20.01- 30	30.01- 40	40.01- 50	50.01- 60	60.01- 70	70.01- 80	80.01- 90	90.01- 100	100.01- 110	
8.01-9	—	4	—	—	—	—	—	—	—	4
9.01-10	—	1	—	—	—	—	—	—	—	1
10.01-11	—	2	—	—	—	—	—	—	—	2
11.01-12	—	—	—	—	—	—	—	—	—	—
12.01-13	—	2	4	—	—	—	—	—	—	6
13.01-14	—	—	—	—	—	—	—	—	—	—
14.01-15	—	2	—	2	—	—	—	—	—	4
15.01-16	—	—	—	—	1	—	—	—	—	1
16.01-17	—	1	3	—	—	—	—	—	—	4
17.01-18	—	—	2	—	—	1	—	—	—	3
18.01-19	—	2	3	4	3	1	1	—	—	14
19.01-20	—	—	4	3	1	2	—	—	—	10
20.01-21	—	—	4	2	3	—	—	—	—	9
21.01-22	—	—	3	5	4	6	—	—	—	18
22.01-23	1	—	2	2	3	4	—	1	—	13
23.01-24	—	—	—	1	—	3	4	1	—	9
24.01-25	—	—	1	2	—	3	3	—	—	9
25.01-26	—	—	1	—	—	—	2	—	1	4
26.01-27	—	—	—	—	—	1	—	—	—	1
27.01-28	—	—	—	1	—	1	—	—	—	2
Total	1	14	27	22	15	22	10	2	1	114

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## DETERMINATION OF CALORIC VALUE OF SMALL SAMPLES

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In studying the nutritional base of fish, the energy balance (especially in fry), the eggs and larvae of a number of fish species, it is difficult and sometimes impossible to obtain samples weighing 1 g (dry weight) required for combustion in a conventional calorimeter. Thus, for example, to obtain 1 g d/w samples one must collect 200,000 specimens of *Ceriodaphnia*, 43,000 specimens of *Daphnia longispina*, etc., i.e. tens and hundreds of thousands of organisms which serve as food for fish. For studies on energy balance, not only the amount of calories taken in with food must be considered, but also the caloric content of the excreta, the number of organisms the fish must feed on in order to excrete 1 g of dry weight of feces should be even higher, depending on food utilization. Hence in a number of instances it is impossible to obtain samples for combustion in an ordinary calorimeter.

For determination of the caloric content of small samples, an indirect approach is often used. This is based on oxidation of organic substances by various oxidants. At one time the method of wet combustion in chromic mixture was widely used. The calculation was done with the aid of oxycaloric coefficient - the amount of oxygen used up for oxidation of the organic substance converted into calories. But even the authors of this method (Vinberg, Ivlev, Platova and Rossolimo, 1934, Ivlev, 1939, Ivlev, 1934) pointed out that a number of organic substances, especially nitrogen-containing ones, are incompletely oxidized in the chromic mixture. Ivlev (1934, 1939) assumed that about 39% of "proteins" are not oxidized to completion. Ivlev recommended to determine the amount of organic substances by means of wet combustion and to determine the total nitrogen content for calculation of the amount of proteins present in the sample, and then to correct for the incomplete protein combustion. Hence this method calls for additional material analyses, and the use of an arbitrary value of 39% of incompletely oxidized proteins.

According to our data (Karzinkin and Tarkovskaya, 1960) the method based on oxidation of organic substances with potassium iodate in the presence of sulfuric acid is more accurate and can be used for the determination of caloric content in small samples. This method is as follows.

A sample weighing about 10 mg (from 8 to 15 mg) is placed in a 300 ml flask connected with a reflux condenser (see Figure) by means of a ground glass joint. The flask should contain exactly 3 ml of a 5% solution of potassium iodate and 20 ml of concentrated sulfuric acid (sp. gr. 1.84). The analysis should be done in duplicates. The control flask contains 3 ml of potassium iodate and 20 ml of sulfuric acid. The flasks are connected with the

condenser without shaking, and heated on an asbestos-covered electric heater in a hood. Boiling is continued for one hour. The condenser must then be cooled. Organic substances are oxidized to final products ( $\text{H}_2\text{O}$ ,  $\text{CO}_2$ ,  $\text{NH}_3$ ,  $\text{H}_2\text{SO}_4$ ) under the action of potassium iodate and oxygen in the presence of sulfuric acid. Oxidation commences immediately during heating, this can be judged by the vigorous release of free iodine which renders the solution a red-lilac. The more oxygen is used for oxidation, the more organic substances are present in the solution, and the more intense the red color of free iodine.

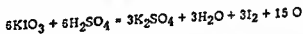


Flask with a reflux for combustion. (Flask is connected with the reflux condenser by means of ground glass joints)

After combustion, the flask is cooled and the transparent and slightly greenish-yellowish liquid is carefully diluted with 50 ml of distilled water. Free iodine is then evolved, rendering the solution a pinkish-orange. The liquid is well mixed (to avoid splashing during heating) and the flasks are heated (not boiled) on an asbestos-covered heater or on a sand bath until complete disappearance of the color and smell of iodine.

After removal of free iodine, the flasks are again cooled and their content diluted in 250 ml of distilled water, 10 ml of 10% solution are then added and the samples are kept in the dark for 10 min.

The remaining potassium iodate is titrated with 0.1 N solution of thiosulfate. It is assumed that potassium iodate is decomposed in amounts equivalent to the amount of oxygen needed for the complete oxidation of organic substances in the sample; Strehlinger (1919) showed that in the absence of organic substances heating of potassium iodate with sulfuric acid to  $200^\circ\text{C}$  does not lead to the decomposition of the former. The amount of oxygen used up is determined from the difference between the amount of thiosulfate used up for titration of the control and experimental flasks. According to the equation:



1 ml of 0.1 N sodium thiosulfate corresponds to 3.567 mg of  $\text{KIO}_3$  and 1 mg of  $\text{KIO}_3$  corresponds to 0.1869 mg oxygen. Multiplying the difference between the amount of thiosulfate used up for titration of  $\text{KIO}_3$  in the control and experimental flasks by  $3.567 \times 0.1839 = 0.66667$  the amount of oxygen used up for the complete oxidation of organic substances in the sample analyzed is obtained.

Having determined the amount of oxygen, one may use the oxy-caloric coefficient equal to 3.38 (Vinberg et al., 1934) and convert the amount of oxygen spent on calories.

Example: 41.08 ml of thiosulfate were used up for the titration of  $\text{KIO}_3$  in the control flask and 25.77 ml in the experimental one.

$$41.08 - 25.77 = 15.29 \text{ ml}$$

$$15.29 \times 0.66667 = 10.13 \text{ mg oxygen.}$$

The sample taken for combustion weighed 10.7 mg, calculation for 1 g of sample  $\frac{10 \times 13 \times 100}{10 \times 7} = 946.7$

$$946.7 \times 3.38 = 3199.8 \text{ g/calorie}$$

This method is sufficiently accurate and simple at the same time. It does not call for special equipment or expensive reagents. A number of determinations may be carried out simultaneously. The high stability of  $\text{KIO}_3$  enables us to store samples after combustion and iodine removal for the determination of the part unspent in oxidation for at least 24 hours prior to titration.

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## METHODS OF INSTALLING CHRONIC FISTULAS IN THE DIGESTIVE TRACT OF FISH

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The rich scientific inheritance of I. P. Pavlov who did so much for our understanding of physiology of digestion in various agricultural animals has been used but little in studies on the physiology of digestion of fish. The classic fistula method has been used only once in fish by A. F. Sulima in 1919. In spite of valuable results obtained by Sulima, this method has not been in use since. This is apparently associated with the difficulties encountered in working with fish and in the absence of suitable books on this method.

### PREPARATION OF PLASTIC FISTULAS

The most suitable fistulas are round or flattened tubes with a protrusion in the middle part and distended fan-like ends. The size of the tube depends on the aim of study and on the size of the fish. It is better that the investigator should prepare the fistulas himself, since he can prepare them according to his requirements.

A very important factor is the weight of the fistula. The fistula should be as light as possible and at the same time robust. Plastics used for artificial teeth are suitable for this purpose.

There are four stages in the preparation of fistulas from plastics: 1) preparation of a wax model, 2) casting the wax model into plaster of Paris, 3) packing with the plastic and 4) finishing.

Preparation of wax models. Pivots of various diameters from stainless steel, brass or copper are used. Having chosen pivots of the required diameter they are coated with wax. One and the same wax model may be used for the preparation of several fistulas of identical size and form, but two pivots of identical size are required for each fistula. Metallic pivots should be smooth and of identical diameter along their entire length, otherwise the fistula will develop cracks during removal from the pivot and will have to be discarded.



FIGURE 1 Wax model of fistula on brass pivot



The wax model is prepared in the following way: First the pivot is covered with a wax plate (commercially available plates used in dental practice) which constitutes the basis of the model of the fistula. The length of the wax plate should be that of the fistula, and its width should be equal to the circumference of the pivot. The plate is warmed slightly on a heater or between the fingers and wound round the pivot. The place where both sides of the wax plate meet on the pivot should be joined by touching it with a hot lancet. If the edges overlap, the excess of wax plate should be cut off and their edges joined. If the edges do not touch, the gap between them should be filled with liquid wax (a piece of wax is melted on a lancet). A wax tubing is thus obtained. Its walls are formed to the desired thickness with a lancet. The middle projection of the fistula is then prepared. To this end a band of wax of the desired width is taken and after softening it by kneading with the fingers, it is attached sidewise on the middle part of the wax tubing. Care should be taken that the projection adheres to the tube. It is then carefully adjusted to the required thickness. In the same way the two terminal rims are prepared (Fig. 1).

It is very important that all joints should adhere closely, otherwise after removal of the model from the plaster of Paris, they may break off and remain in the depressions of the plaster of Paris.

The metallic pivot and the wax model are then coated with a thin layer of vaseline by means of a brush.

CASTING THE WAX MODEL IN PLASTER OF PARIS. The wax model is cast in plaster of Paris in a detachable mould (cuvette used by dentists) consisting of two halves with a removable lid. First one must learn to prepare plaster of Paris of the required consistency of sour cream. Our experience is that for filling two moulds (cuvettes), 400 ml of plaster of Paris and 240 ml of water should be used for the lower halves, and 300 ml of plaster of Paris and 180 ml of water for the upper halves. This is only an approximate proportion, since the quality of plaster of Paris, and especially its moisture content, may vary.

Plaster of Paris is diluted in a large crucible. The diluted plaster of Paris is cast into the lower half of the mould, the surface being smoothed to the level of the edges of the cuvette. Excess plaster of Paris is removed. When the plaster commences to solidify, one half of one or of two wax models with pivots are immersed in it. The roughened surface of the plaster of Paris should be smoothed out with a lancet. When plaster of Paris solidifies (to enhance its solidification the mould may be placed on a heated surface) its entire area and the surface of the wax model jutting out from the plaster of Paris is coated with a thin layer of vaseline by means of a brush. The lower half of the mould is covered with the upper half and plaster of Paris is cast inside to full capacity. It is very important that all grooves of the wax model should be filled with plaster of Paris. For this purpose a small amount of plaster of Paris is cast, the mould is shaken and then filled to capacity with plaster of Paris. When the plaster of Paris begins to solidify the lid is fitted in place and the mould is placed under press. This removes the excess of plaster of Paris and both halves of the mould and the lid are tightly sealed. The mould is then taken out and left overnight. Plaster of Paris in the mould should solidify well.

PACKING WITH PLASTIC. The upper half of the mould is taken off, being separated from the lower part by means of an awl or screwdriver.

The model of the fistula remains in the plaster of Paris of the lower half of the mould. The metal pivot with the wax model of the fistula is carefully taken out, for this purpose the pivot should be hooked and lifted with a sharp tool.

The metal pivot with the wax model may be stored (and repaired if damaged during the procedure). It may then be used for the preparation of another fistula. If the fistula rims remain in the plaster of Paris, wax should be removed by pouring hot water over it to dissolve the wax or it may be cut out with a lancet. The plaster of Paris of each half of the mould contains depressions corresponding to the half of the model of the fistula.

Plastic is then prepared (AKR-7 or veronite may be used). Both are used for the preparation of artificial teeth. The powder should be mixed with the liquid in a small porcelain crucible by means of a glass rod. The mixture should be mixed until it can be stretched. The mass obtained is divided into two halves, each half is rolled into a pancake, and squeezed by hand into the depressions in the plaster of Paris, each half of the mould is filled separately. The plastic mass in the lower half of the mould (cuvette) is covered with a metallic pivot of the same size and form as that used for making the wax model (hence two pivots are required for each fistula). If only one pivot is obtainable, the first pivot may be used after removal of the wax.

The lower part of the mould is covered with a cellophane sheet which is slightly moistened in water and covered with the upper half of the mould. The entire mould is put under pressure. The latter is tightened until both halves join closely. Then the mould is removed from under the press, the two halves are separated, and the cellophane is removed and again put under the press. Afterwards the mould is placed in a container which is tightened and placed in boiling water (or electric sterilizer for 30 min). The whole mould must be completely immersed in boiling water (if not, it must be turned over after 15 min).

If veronite is used, the entire procedure must be carried out as quickly as possible, otherwise the veronite dries, loses elasticity, and the fistula becomes brittle.

The special container with the mould should be removed from the boiling water and left overnight.

**Finishing.** Next day (or earlier - it is important that the mould should cool) both halves of the mould are separated with the aid of an awl, lancet, or knife, and the fistula is taken out. Plaster of Paris is removed from the mould, and the latter is washed and dried. The fistula should be separated from the pivot by simply knocking it out of the latter. This should be done carefully, otherwise the fistula may split.

The excess of plastic is removed by means of a file. The fistula should assume the appearance of the wax model. The surface is smoothed by fine files and polished on a special carborundum disk used by dental technicians (the disk is set in motion by a small motor). Afterwards the fistula is polished with felt and a ring-shaped brush. One can also use felt with bricks moistened with water.

If the veronite fistula is turbid-white and brittle instead of glass-like and transparent, it means that too little liquid was added to the powder or that during mixing the moment of optimum consistency was missed, and the plastic has become too dry.

The following apparatus and materials are required for the preparation of plastic fistulas: 1) mould press, 2) copper mould; 3) double or single container, 4) electric motor, with 5) rheostat; 6) polishing brush; 7) felts; 8) brass or copper pivots, 9) wax (as used in dental practice); 10) veronite or AKR-7 plastic, 11) sterilizer, 12) plaster of Paris; 13) metal spatula, 14) small and large lancets, 15) graduated cylinder, 16) small and large crucibles, 17) cellophane; 18) enamel mould (euvette); 19) hammer; 20) screwdriver; 21) ground brick; 22) various files; 23) alcohol burner; 24) small, soft brush, 25) vaseline; 26) carborundum disks (dental type).

## SURGICAL PROCEDURES ON THE DIGESTIVE TRACTS OF FISH

### Preparation of Operation

In order to be able to operate on fish, a thorough knowledge of the structure of the digestive tract of the species under study is necessary. This includes the location of inner organs in the body cavity, as well as the distribution of large blood vessels and nerves along the digestive tract. A projection of inner organs onto the body surface should be made and reference points drawn, according to which the experimenter will be able, during the operation, to select the site of the incision for the insertion of fistulas in the desired region of the digestive tract. The most suitable reference points are the pectoral and ventral fins. The arrangement of the digestive tract in the carp is illustrated in Fig. 2. The figure indicates the points at which it is convenient to insert the fistulas in different parts of the digestive tract.

The fish should be prepared for the operation. Fish brought to the laboratory from a water reservoir are at first so terrified and excited that they may jump out of the aquarium and refuse to take food. Some of them may be injured during the transportation, or may be ill and not infrequently perish. It takes from several days to one - two weeks to adapt them to laboratory conditions. It is important to get the fish accustomed to nutrition under the new conditions. After the fish have adapted to life in the aquarium and commence to search actively for food, they can be operated on. Surgical procedures are best performed when the digestive tract is empty. For this reason the fish should be starved for 2 - 4 days before the operation.

Before the operation the fish should be anesthetized by placing it in a 2% aqueous solution of ether until reflexes disappear. The fish should be kept in ether solution for 5 - 10 min, depending on its size and on the freshness of the solution. The fish is then taken out of the ether solution and placed in an ordinary container or on a specially designed operation table. The fish should be wrapped in wet gauze, the operation field should be exposed. Scales at the site of the planned incision should be pulled out with forceps. No other preparation of the operation-field is required.

The usual instruments, including needles and sutures, are used. The following surgical instruments are required: various scalpels, anatomic and surgical forceps, large and small blunt-tipped and pointed scissors, a grooved probe, blunt hooks for wound stretching, needle holder, various needles, etc. Among special instruments required mention must be made

of 2 or 3 blunt metal hooks for pulling out the stomach and intestines. In our work, a thin capron\* suture was most satisfactory. No sterilization of instruments, materials and hands of the surgeon is required.

The surgical field should be well lit.

The size of the fish and the structure of their digestive tract vary widely. Hence one must prepare several fistulas of varying diameter, size length and form, to fit each specimen.

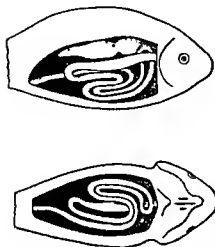


FIGURE 2 Location of digestive tract in the body cavity of the carp

#### PREPARATION OF GASTRIC FISTULA

The site and exact point of incision must be decided on before the operation. Incision through the skin and muscles is done with pointed scissors (better in transverse direction in relation to the fish body). The size of the incision should be as small as possible, so that only the stomach can be pulled out through the opening. For example, in the case of dwarf (*Ameiurus nebulosus*) and common sheathfish (*Silurus glanis*) weighing 150-200 g the length of the incision is 0.8-1.0 cm. First the skin is incised (it can easily be held with forceps), this is followed by an incision through the muscles and finally through the visceral

peritoneum. The stomach is pulled out with a blunt hook. Care should be taken that the other internal organs are not damaged in the process (gallbladder, liver, swimbladder and others) and that large blood vessels are not torn, large blood vessels in fish, in contrast to warmblooded animals, may sometimes lie on the surface of the stomach and can be easily separated from the adjoining tissue.

The site of incision into the stomach is then decided on. Owing to the location of large blood vessels and nerves, it is advisable to make the incision close to the greater curvature, between the blood vessels and parallel to them. Care should be taken not to injure the blood vessels in the process. Then a purse-string suture is made which delineates the segment of the future incision. In the case of fish weighing 150-200 g the incision should be approximately 0.4-0.5 cm long. The smaller the incision the better, because the stomach wall stretches easily during installation of the fistula. When the stomach is large, the suture should not pass through the mucosa (only through the serous and muscle layers). Subsequent incisions should not touch the mucosa. The muscle layer is separated slightly along the edges, the submucous and mucous layers are cut through with scissors, a purse-string suture is made, the fistula is inserted and both purse-string sutures are tightened and tied with a surgical knot (Fig. 3). The edges of the wound are turned in.

\* [Soviet name for polycaprolactum resin and fiber]

The serous-muscular layer may be cut without a preliminary purse-string suture. In this case, after having cut through the muscular layer, its edges must be slightly separated and the purse-string suture is used on the muscles. Subsequent procedure is similar to that described above. If blood vessels are damaged and hemorrhage commences, it should be arrested by ligature. Blood vessels which cannot be bypassed during the incision should also be ligated.

The stomach is now cleared of blood clots and tissue fluids with a weak  $\text{KMnO}_4$  solution. Then the stomach together with the fistula is carefully replaced through the cut, and returned to its normal position in the abdominal cavity. The fistula should be inserted in such a manner that its middle rim should be in the abdominal cavity.

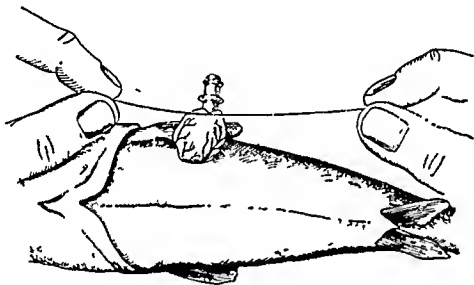


FIGURE 3 Tightening of purse-string suture around the fistula in the stomach of a dwarf sheathfish

If the incision through skin and muscles is too large, the fistula should be held by one or two simple sutures. These sutures must be very sturdy. It should be kept in mind that all sutures irritate the surrounding tissues to a certain extent, and may cause necrosis of the tissue around the fistula (this is especially frequently noted in carps). Hence, the incision through skin and muscles should be as small as possible and any superfluous sutures should be avoided. Tightening of the purse-string sutures must also be done carefully, and similar precautions should be taken throughout.

The fistula is stoppered with a plug prepared for this purpose beforehand.

When small fish are operated on, all layers of the stomach wall may be cut through with a single stroke. The muscle layer along the edges of the incision is carefully separated from the mucous and submucous layer, and two purse-string sutures are made, one on the mucous layer and the other

on the muscle layer, the fistula is inserted, and the sutures tightened and tied with a surgical knot.

The fistula must clearly fit the size of the fish operated on, it should not be too wide or too long. Round fistulas are preferable

The operation should be completed within 15 - 20 min.

#### PREPARATION OF ISOLATED STOMACH

This operation (especially on small fish) requires experience and skill in surgical procedures. It has been carried out with success.

In installing of isolated (pouch) stomachs, one should be guided by the technique of a similar operation carried out on dogs (technique developed in I. P. Pavlov's laboratory). The stomach is pulled out of the abdominal cavity, the site of incision and its size are determined, the latter depending on the size of the stomach. If the stomach is small a larger incision must be made, otherwise the final isolated stomach will be too small.

The incision should be parallel to the greater curvature. During this procedure, attention should be directed mainly to preservation of the nerves and local blood vessels. To avoid hemorrhages all large vessels (vascular branches) along both sides of the incision should be ligated (on the anterior and posterior stomach walls), both stomach walls are then incised. The stomach walls may be cut through with a single incision, or preferably layer by layer. First the muscular layer is dissected. This is followed by an incision through the mucous and submucous layers. Prior to incision through the mucous and submucous layers, it is better to separate the muscles along the edges of the incision (this facilitates further separation of the mucous isthmus). As a result, part of the future cardiac pouch is isolated and connected with the main part of the stomach through the isthmus through which all main blood vessels and nerves pass.

The next stage - the most critical and difficult stage of the operation - consists of isolating both gastric segments (the main portion and the cardiac pouch) forming a wall and a vault from the mucous layer and then forming the stomach and the pouch as a whole. For this purpose the mucous and submucous layers of the isthmus are separated along the planned incision, and the incision is made only through these two layers. After cutting the layers of the isthmus, they are separated a little along the edges of the incision along its entire length in the direction of the main stomach and in the direction of the pouch. This must be done very carefully so as not to damage the nerves and blood vessels passing through the underlying muscle layer. The mucous layer is then sutured to the submucous layer of the main stomach, commencing from the vault and to the end of the incision. In a similar way the mucous and submucous layers of the isolated gastric pouch are sewn together. The only difference is that on its distal end an opening is left, into which the gastric fistula is inserted. The mucous and submucous layers are sewn with their edges turned inwards. The muscle layer of the stomach and that of the cardiac pouch are then sewn together, and the edges of the incision are also turned inwards. To avoid any communication between the stomach and the pouch, the vaults are prepared very carefully with two suture layers.

Insertion of the fistula into the isolated gastric pouch is carried out by the method described above.

The operation should be completed within 50 - 60 min. A schematic representation of the operation of an isolated gastric pouch in the common sheathfish is illustrated in Fig. 4

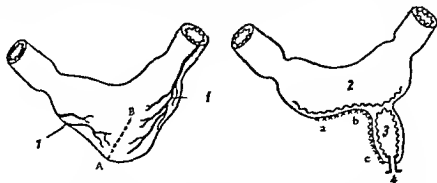


FIGURE 4 Isolated stomach in common sheathfish, diagram on left - stomach before the operation on right diagram - after the operation

AE - line of incision, abc - line of sutures, 1 - nerves, 2 - cavity of the main stomach, 3 - cavity of the isolated stomach, 4 - gastric fistula, the wavy line (indicates the mucous layer (diagram on right))

#### PREPARATION OF INTESTINAL FISTULA

A convenient place in the selected segment of the intestines is first chosen (depending on the aim of the procedure). The skin and muscles are incised by the method described above, and the intestinal loop is pulled out by means of a blunt hook. It should be remembered that the loose tissue of the intestinal wall, especially in small fish, can be easily torn, and that the diameter of the fish intestine in smaller species is very small. For this reason, a purse-string suture must be placed on the segment delineated for incision. The segment within the suture should be oval, with its long axis along the length of the intestine; its size should be such as to include the entire length of the incision.

The cut through all layers is made with a single incision. Hemorrhages should be avoided during the incision through the intestinal wall and during the suturing. The fistula is inserted through the incision, and the purse-string suture is pulled tightly and tied with a surgical knot around the fistula. If the suture is not tight, another should be made. Superfluous sutures should be avoided, tightening should be done carefully, etc. The intestinal loop with the fistula is carefully replaced in the abdominal cavity, if possible at its former site. The incision through the muscles and skin is sutured in the way already described.

The preliminary purse-string suture on the intestinal wall made before the incision may be dispensed with. In this case the procedure is similar to that described for the operation on the stomach in small fish. Flat fistulas are preferable.

The operation should be completed within 15 - 20 min.

#### PREPARATION OF TWO FISTULAS

Two chronic fistulas may be inserted in the course of one or two operations. In the latter case, the second fistula is inserted several days after the first fistula, when the fish has recovered from the first operation. If two fistulas are to be inserted in the course of one operation, the duration of the anaesthesia should be prolonged by nearly  $1\frac{1}{2}$  times.



FIGURE 5. Dwarf sheathfish with two fistulas (in stomach and in intestines).

In Fig. 5 a dwarf sheathfish weighing 220 g. with two fistulas (in the stomach and in the intestines), is illustrated. The two fistulas were inserted in the course of a single operation. No postoperation complications were observed. The sheathfish was under observation for 106 days.

#### POSTOPERATIVE PERIOD

After operations the fish should be carefully nursed. They should be carefully inspected each day. They should be placed in optimal conditions as far as air, mineral composition of water, temperature and food are concerned. It is advisable to wrap aquarium walls with a thick paper to avoid jerky movements on approach of man. After operations the fish can be immobilized in special devices (this was practiced by A. F. Sulima (1919), who after operations kept the fish in such devices in the aquarium). According to our experience some fish, such as carp and tench, do not survive long in an immobilized position and die after several days, even without the operation. Thus we avoided long immobilization periods in these species. As to other fish species, immobilization is probably recommended. In addition,



some physiological experiments call for immobilization of the fish. For this reason we shall describe a special device of this type used for fish immobilization.

We have prepared certain apparatus similar to those used by N. Belousov (1900) and Baglioni (1908) for studies on respiration and physiology of swim bladders in fish.

The device is prepared from metal plates in the shape of a fish body, and consists of two folds which can be locked after the fish is placed inside. Transverse beams should be quite close so that the fish will not be able to slip out. At the same time they should not hamper fish respiration. The device is suspended on metal pivots and a wooden beam. When the device is placed in the aquarium with the fish, the wooden beam may float on the surface or lie on its walls. We kept fish in such immobilizing devices in natural water reservoirs. We then attempted to construct devices to keep fish in aquariums so that they would be able to live under normal conditions and so that the investigator should be able to take them out whenever he wishes to examine them or to submit them to certain tests.

The method by which fish with gastric fistulas are taken out of the aquarium is very important, if they are taken out carelessly they may lose their fistulas. Fish can be taken out by means of a landing-net, the following conditions must be observed:

- 1) the landing net should be made of a very fine net (coarse gauze) so as not to entangle the fistula,
- 2) each fish should be caught carefully, and no jerky movements are allowed,
- 3) each fish should be taken out of the net by the assistant, who should take it in both his hands (to keep it from struggling), and in this process the fistula should not be touched, but should be freely suspended.

If these instructions are not followed the valuable experimental fish may perish.

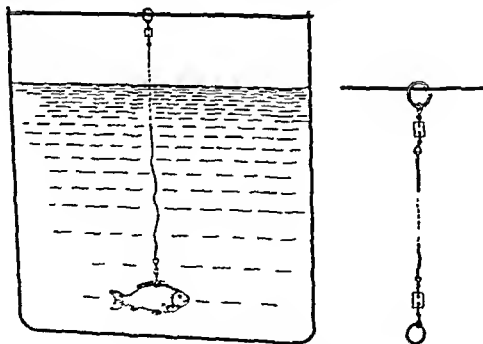


FIGURE 6 Fixation of fish with fistula in aquarium

After having tried several different methods, we chose the following one. An ordinary fishing line (its thickness depends on the size of the fish, and its length on the size of the aquarium) is threaded (with the aid of a surgical needle) through the muscles of the back of the fish, under the base of the dorsal fin, while the other end of the fishing line is tied to a fishing carbine with a ring through which a metal wire passes. The metal wire is suspended above the aquarium. The movements of the fish are unrestricted, while the investigator can take the fish out easily and gently whenever he so desires. When the fish is taken out of water it should be supported by the second hand and wrapped in moist gauze. This can be done easily after some training. This method of fixation of fish is very successful not only with fish which are kept in an aquarium, but also with fish kept in special containers in natural water reservoirs (Fig. 6).

What complications may occur in the postoperative periods?

According to our experience, based on post-mortem examinations, the following reasons of death in the experimental fish have been revealed,

- 1) necrosis of tissues around the fistula and prolapse of the fistula (when heavy fistulas are used),
- 2) damage to the gallbladder,
- 3) damage to large blood vessels with formation of hematomata,
- 4) improper fixation of the fistula in the gastric or intestinal walls, as a result of which the fistula may fall out,
- 5) paralytic ileus (during or after the operation).

Post-mortem examination of all fish should be made so that the errors made during the operation are revealed. Let us mention that the data on the fish to be operated, as well as the entire course of the operation, should be recorded in a protocol.

Experiments on fish with a fistula should be commenced after the incision through the skin and muscles has healed and the fistula is firmly held in place. This occurs usually 10 - 15 days after the operation.

If the operation is successful and no complications develop the fish with the fistula may live long and normally.

#### FEEDING OF FISH WITH FISTULA COLLECTION OF FOOD MASS AND DIGESTIVE JUICES

#### FEEDING FISH WITH CHRONIC FISTULAS OF THE DIGESTIVE TRACT

One must distinguish between special and general feeding of fish with chronic fistulas of the digestive tract. Both depend on the aim of the investigation in question. Active food intake by fish after the operation depends on three factors. First, the fish should get accustomed to life in the aquarium and to active feeding before the operation, second, no postoperative complications must occur; third, life conditions in the aquarium should be normal and not interfere with metabolism of the fish (size of the aquarium) the water temperature, gaseous interchange, etc.)

From the moment one commences to feed the fish with the fistula (not before 3 - 5 days after the operation), the fish must be watched whether it

takes the food and whether it passes feces. According to our observations most sheathfish begin to feed actively 4-8 days after the operation, and carp 10-12 days after the operation. Experimental fish should be weighed periodically, since weight gain is the best criterion for quantities of food eaten.

When fish are kept on an ordinary diet, it is sufficient to put adequate food into the aquarium. If to all appearances no complications have occurred, but the fish nevertheless loses weight, it can be fed artificially by giving food through the mouth or administering it directly into the digestive tract through the fistula. If food is returned it can be introduced on a thread fixed in the fistula. According to our observations this method stimulates the digestive processes, and fish so treated commence their active search for food. In the course of further experiments, the fish may be fed by adding the food in the aquarium or by placing food in the mouth of the fish or directly into the desired segment of the digestive tract through the fistula.

If food is placed in the aquarium, the amount of food taken by the fish is calculated by the food which remains. If the fish is fed directly into the mouth, then of course the amount of food taken is known. However, such feeding precludes the search for food, and the catching and swallowing of it, all processes of great importance for digestion. For studies on the role of different segments of the digestive tract in digestion, the food is introduced through the mouth or through a fistula inserted into the corresponding segment of the digestive tract.

One of the criteria of the beginning of feedings and digestion after the operation is the appearance of mucous excrements. Some time after the appearance of mucous excrement, normal feces usually appear.

Fish with fistulas in the middle and posterior (especially in the posterior) segments of the digestive tract (stomachless fishes) and of intestines (gastric fishes) are the first to commence active feeding after the operation.

#### COLLECTION OF FOOD MASSES AND DIGESTIVE JUICES

The content of the digestive tract and of the digestive juices in fish with chronic fistulas can be collected in the following way.

a) the fish is taken out of the aquarium, the fistula is opened and the content of the digestive tract is removed through the fistula into a vessel.

b) the fish is taken out from aquarium and the food mass and digestive juice are collected with the aid of a pipet. This method is suitable for collecting liquid food masses and digestive juices

c) food is taken out from the fistula with the aid of forceps, a grooved probe, or a special small metallic spatula. This method is suitable for collecting food masses from intestines of predatory fish and from the middle and posterior segments of the digestive tract in stomachless fish

d) digestive juice and food masses are collected in a special receiver (a rubber receiver is preferred) which is tied to the external rim of an open fistula and with which the fish must live for some time and move freely, and which ensures a normal way of life for it. The bulb of a glass pipet may serve as a receiver.

e) small amounts of liquid food and digestive juice may be collected by means of a weighed filter paper (the juice moistens the filter paper)

Concerning the collection of pure digestive juices unmixed with food and secreted in the course of digestion, the isolated stomach method is one of choice. We have succeeded in carrying out some observations (after succeeding in performing this operation) on the secretion of pure gastric juice in the course of digestion and on its reaction in common and dwarf sheathfish

Pure gastric juice can be obtained by mechanical stimulation of the gastric mucosa by some indifferent stimulants (introduction into the stomach of some glass or rubber "beads" on a thread through the fistula).

No methods are available as yet for the obtaining of pure intestinal juices from living fish in long-term experiments. The method developed by V. A. Pegel' (1958) for studies on the mechanism of secretion of pancreatic juice in a Siberian dace is promising

Chronic fistulas have been used successfully for gaining access to various segments of the digestive tract of fish in repeated experimentation. It has been used for the following purposes:

- 1) studies on the passage of food and changes which the food undergoes in the course of digestion,

- 2) studies on chemical changes of food,

- 3) studies on the site of food absorption,

- 4) studies on the mechanism of secretion of digestive juices and on the properties of these juices;

- 5) studies on the effect of various factors on the intensity of digestive processes.

The important feature of this method is the possibility of long-term studies of a normally functioning digestive tract under natural conditions (in an aquarium or fish pond) and active feeding.

This method may also be used for studies on the physiology of other organs.

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# THE USE OF METT'S RODS FOR THE SIMULTANEOUS DETERMINATION OF ENZYME COMPOSITION, ENZYME ACTION AND MOTOR ACTIVITY OF THE DIGESTIVE TRACT IN FISH IN CHRONIC EXPERIMENTS

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For simultaneous studies of enzyme composition, enzyme activity and motor activity of the digestive tract in fish in chronic experiments, so-called Mett's rods may be used, these were recommended by S.G. Mett in 1889 during his work in I.P. Pavlov's laboratory. Apart from the known protein and carbohydrate rods used for *in vitro* studies on the activity of proteolytic and amylolytic enzymes of the digestive juices, one may also use fat rods for the determination of lipolytic enzymes in fish in chronic experiments.

For preparation of these three types of rods, thin-walled capillaries about 2 mm in diameter and 20 cm long are used, after filling them with the corresponding substrates by means of suction.

Protein rods are prepared in the following way. Fresh egg white free of yolk is collected in a porcelain crucible and freed from membranes by separating the egg white with scissors and filtering through a double layer of muslin. The egg white is then mixed with physiological salt solution (egg white-salt solution, 9:1 v/v). The mixture is filtered through filter paper for a day. For the removal of air bubbles the filtrate is placed in a desiccator under reduced pressure (achieved by means of a vacuum pump), 15–20 hours later the egg white is taken out of the desiccator and drawn into capillaries which are immediately immersed in hot water (90–95°C). The rods are left there for 5 minutes, their ends sealed with Mendeleev paste and stored in a vessel filled with water. The protein rods are ready for use in this form.

Protein rods may also be prepared from blood plasma. They are easier to prepare and better digested. Blood is taken from the carotid artery of a dog or rabbit (blood can also be obtained from a rabbit's ear). The blood is centrifuged for 3–5 minutes. Plasma is drawn by suction into the capillaries and coagulated in hot water. The rods are prepared beforehand and are stored in the same way as those prepared from egg white. Gelatin rods may also be prepared (gelatin may serve as a substrate).

Starch rods are prepared by drawing up into the capillaries a hot mass consisting of a 4% aqueous solution of starch with some amount of methylene blue, this mass is heated and stirred constantly with a glass rod. Methylene

blue serves for delineating the boundary of digested starch. The rods are sealed with Mendeleev paste and stored in cold water. They may be stored for 2-3 days.

For the preparation of fat rods, fat which is solid at 30-35°C may be used, such as lard. This is digested with difficulty, however Margarine is thus preferable.

Immediately before the experiment the rods are cut into small pieces 0.5-0.6 cm long, the edges rounded off by the file used for cutting the rods to pieces, and placed on a watch glass filled with water (to prevent drying up of the surface of the substrates). The rods are then inserted into the digestive tract of the fish. For this purpose forceps with grooved flat tips are used, or a specially designed syringe-like glass tube, the inner diameter of which should be slightly smaller than that of Mett's rod. The tip of the tube should taper slightly to facilitate its introduction into the esophagus.

A thin wire piston with a 'restrictor' is placed inside the tube so that the piston will protrude from the lower opening of the tube by not more than 1 mm (Fig. 1).

Before inserting the tube the piston is slightly raised, and the rod with the appropriate substrate is placed in the lower part of the

glass tube. The fish is then taken out from the aquarium with the left hand, and with the right hand the narrow end of the tube is inserted into the mouth of the fish.

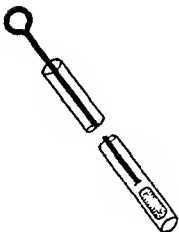


FIGURE 1 Glass tube for inserting Mett's rods



FIGURE 2 Introduction of Mett's rod into the anterior segment of the digestive tract of fish

By pushing the piston the rod is drawn through the esophagus and into the anterior part of the intestines (Fig. 2). The fish is then placed for 10 - 15 minutes in a separate vessel filled with water to check whether the rod has been introduced into the intestines. Such a control is essential, since sometimes the rod may be pulled back and remain in the mouth of the fish. Some training is needed for the successful introduction of Mett's rods into fish intestines. Unexperienced persons may perforate the intestines with the forceps.

The exit of the rod from the anal opening is recorded, in other instances the fish is killed after a given time, dissected and the localization of the rod in the intestines established. The time of retention of the rod in the intestines serves for assessing the motor activity of the digestive tract. The depth to which the substrate in the rod (taken out of the intestines or excreted) has been digested is measured with a ruler and magnifying glass.

To study the effect of starvation, and of the quality and quantity of food on the motor activity of the intestines, as well as on the activity of enzymes of the intestinal juices, it is necessary to feed the experimental fish actively or by artificial feeding before introducing the rod. Artificial feeding consists of the introduction of a known amount of food into the anterior segment of the intestines by means of tweezers or other instruments. For studies on the effect of certain external factors, in particular that of temperature, on the motor activity of intestines and the activity of intestinal juices, the fish should be examined under suitable conditions, for example in aquariums with different temperatures. The rod is introduced into the anterior segment of the intestines and the fish is kept under the specified conditions until the rod is excreted through the anal opening.

In the same way studies can be carried out on the effect of other factors, (such as damage to the nervous system and various pharmacological substances) on the digestion of fish.

This method can be used for simultaneous studies on the enzymatic and motor activity of the digestive tract in stomachless fishes only, because the rod may be retained in the stomach and the time of its retention in the digestive tract, all conditions being equal, may vary widely. In the case of fish with a stomach, motor activity and enzymatic activity should be studied separately.

By means of this method it has been established that fishes have proteolytic, amylolytic and lipolytic enzymes throughout the entire digestive tract. The digestive activity of these enzymes as well as the peristaltic movements of the intestinal muscles depend on various factors, especially on temperature, the amount of food in the tract, and others.

## METHODS OF STUDY OF THE DIGESTIVE PROPERTIES OF PANCREATIC JUICE AND BILE IN FISH IN LONG-TERM EXPERIMENTS

V. A. Pegel'

(Tomsk State University)

For studies on the digestive properties of pancreatic juice in stomachless fishes with isolated anterior segments in long-term experiments, it is necessary (see Fig on p. 142) to introduce Mett's rods (capillaries) with protein, carbohydrate and lipid substrates into the oral cavity of the fish. The enzymatic composition, and the activity of pancreatic juice enzymes, is assessed by the amount of the substrate in the rod which undergoes digestion after the rod had been excreted from the distal end of the duodenum which has been exteriorized for this purpose onto the skin.

To determine the role of bile enzymes passing into the duodenum together with the pancreatic juice through the bile duct, bile must be collected from several gallbladders and its effect tested with the various substrates in Mett's rods in *in vitro* experiments. The enzymatic composition and enzymatic activity of the bile is assessed from the amount (millimeters) of protein carbohydrate and fat digested. In Siberian dace, for example pancreatic juice contains proteolytic, amylolytic and lipolytic enzymes, whereas the bile is devoid of all digestive enzymes.

To examine the role of bile in digestion in fish in long-term experiments the passage of bile into the intestines must be prevented by tying the bile duct. This operation in daces is described.

The fish is taken out of the water and wrapped in a wet towel so that it will not compress its gill covers. The operation is performed without anesthesia. Surgical instruments are sterilized by immersing them in alcohol. Our experience showed that strict aseptic or antiseptic measures are unnecessary in this operation. The fish is immobilized and an incision is made into the abdominal cavity with a scalpel or scissors. By means of pincers, the tips of which are bent at almost right angle, two threads are placed under the anterior segment of the intestines (2 cm from its esophageal part). With the aid of one thread held on both ends the intestine is lifted, while the end of the other thread, nearer to the surgeon, is held by the forceps and wound around the intestine in its foremost part adjoining the esophagus. This is facilitated by lifting the intestines with the first thread. Thus the second ligature passing under the intestine in two places is located at both sides of the bile duct covered by the liver. Only a small part of the gallbladder is visible under the



liver. The loop thus formed is located along the intestine, extending into a small slit between the longitudinal part of the intestine and liver which covers it. The free ends of this loop on the other side of the intestine are tied in a knot. When this is tightened the loop goes deeper into the space between the liver and intestine, and separating it from the latter reaches the duct of the gallbladder and tightens around the duct near its entrance into the intestine. Another knot is made, and the free end of the ligature is cut off. The operation is completed by sewing up the abdominal cavity with a continuous suture. A schematic representation of the ligature on the gallbladder duct in dace is illustrated in the Figure.

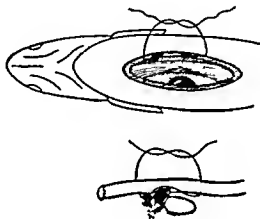


FIGURE. Ligature on gallbladder duct of dace.

Immediately after the operation the fish should be transferred into an aquarium. For about one hour the fish swims on its back and then assumes its normal position and cannot be differentiated in its behavior from the other, intact fish.

A certain amount of training is needed for the successful completion of this simple operation. Daces operated on in this manner have lived for 7-15 days. They gradually develop an icterus which is easily diagnosed by the increasing yellow color of the white abdomen. Several days later the abdomen of the fish commences to grow, reaching considerable size before the fish dies.

Post-mortem examinations have shown that congested mucous masses accumulate in the anterior segment of the intestine, and do not move along the intestine because of atonia of the intestinal walls due to the lack of bile. In the same place one can find Mett's rods introduced on the second day after the operation. Under normal conditions these rods are rapidly propelled along the intestines due to the peristaltic movements of the latter.

The extent to which the substrates of the rods are digested, especially the fat, is markedly decreased, which indicates that the bile activates the pancreatic, and especially the lipolytic enzymes.

# STUDIES ON ENZYME COMPOSITION, ENZYME ACTIVITY AND MOTOR ACTIVITY IN VARIOUS SEGMENTS OF THE DIGESTIVE TRACT OF FISH IN LONG-TERM EXPERIMENTS

V.A. Pegel'

(Tomsk State University)

Studies on enzyme composition, enzyme activity and motor activity in various segments of the digestive tract of fish by means of Mett's rods involve surgical interventions aimed at isolating various segments of the digestive tract. The simplest method is to kill the fish and to dissect the digestive tract a certain time after the introduction of Mett's rod. Depending on experimental conditions, the rod will reach a certain segment of the intestine. Knowing the time elapsed since the introduction of the rod and the distance the rod has travelled, the motor activity and enzymatic activity of the segment traversed by the rod can be determined. Another method involves the placing of ligatures on the segments to be studied. A small incision is made, the rod is introduced into the segment to be studied, and this segment is separated by ligatures. The rods may be introduced through the mouth into the duodenum, which should be ligated distally. After a given time interval the fish is killed, dissected, and the rod taken out. The digestion of substrates present in the rod is then determined. Food may be introduced into the intestines together with the rod (piece of meat, bread, etc.).

The duration of the operation is several minutes, no anesthesia is required. The operation does not impair the well-being of the fish (dace, carps). 10-15 minutes after the operation they swim normally and can be used for experiments in the course of 24 hours. The drawback of this method is the short duration of the observations, and the inability to repeat experiments on one animal. In addition, only enzyme composition and enzyme activity can be determined; simultaneous studies on motor activity of the intestines are not possible.

An improved method without the above drawbacks consists of the following (operation on Siberian dace is described):

The fish is taken out of the water and wrapped in a wet towel. An incision is made along the abdominal part, 0.5 cm proximal to the anal fin. The incision through the skin should be 1.5 cm long. A small segment of the duodenum is pulled out by means of pincers with tips bent at a right angle. The intestine pulled out should adjoin the region of the first intestinal loop. The operation then proceeds in one of two possible directions. In one case a small transverse incision is made into the intestine, through the edges of

which threads are sewn, these serve as "handles" for holding the ends of the intestine. Then in place of the transverse cut the intestines are separated by cutting through them. Both ends are separately sewn to the skin, and the free space between them is covered with skin. A preparation resembling a Tiri-Wella intestinal fistula is obtained (Fig. 1).

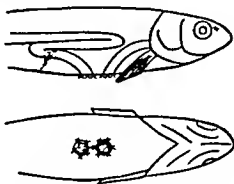


FIGURE 1. Complete isolation of the anterior and middle intestinal segments in dace

In another case a 1 cm long longitudinal cut is made into the intestinal wall. The edges of this cut are sewn to the wound by separate sutures. Two additional openings are formed, which divide the intestine into a short cranial part including the gallbladder and pancreatic ducts, and a long caudal part, between them a muscle bridge remains (Fig. 2).

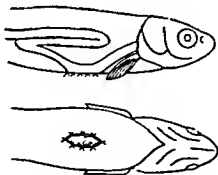


FIGURE 2 Partial isolation of the anterior and middle segments of the intestine in dace

Each operation takes 10 - 15 minutes. Dace bear it well. Frequently immediately after operation the fish assume a normal posture and do not differ in their behavior from other fish in the aquarium. Such fish may live for

months and eat well. They can serve as experimental subjects for studies on enzyme composition, enzyme activity and motor activity of each of the isolated segments of the digestive tract. For this purpose, rods with proteins carbohydrates and fats are placed into the anterior segment (through the mouth) or into the cranial segment of the intestine sewn to the skin, the time when they are excreted and the extent of digestion of the substrates in the capillaries is recorded and measured (in millimeters).

It was shown that in dace the anterior segment exhibited proteolytic, amylolytic and lipolytic activities, but the posterior segment was shown to have only an amylolytic activity. The motor function of the posterior segment depends on its link with the anterior segment. Experiments on dace (operated on by the two methods, see Figs. 1 and 2) showed that the anterior segment stimulated peristaltic movements of the posterior segments of the intestines.

## METHODS OF OSMOTIC REGULATION STUDIES IN FISH

E.A. Veselov

(State University of Petrozavodsk)

The choice of a method for studies on osmotic pressure depends on the subject, experimental conditions and aims of the investigation, and primarily on the amount of biological liquid available. The most accurate and widely used method at present is the cryoscopic method based on the capacity of osmotically active substances to lower the freezing point of the solution, which is proportional to the osmotic pressure of the solution. The cryoscopic method is, however, not suitable for work under field conditions.

### OBTAINING OF BLOOD SAMPLES FROM FISH FOR OSMOTIC PRESSURE DETERMINATION

Samples of blood, lymph and tissue sap fluids which form the internal medium of the organism required for osmotic pressure measurement must fulfil several conditions. Certain precautions must be observed when the samples for studies on osmotic pressure are obtained, precautions which are superfluous in the case of samples taken for chemical and morphological studies.

The sample must be pure, uncontaminated by mucus and other substances. During the taking of samples the osmotic pressure of the latter should remain unchanged. Fish blood coagulates easily. Anticoagulants such as citrate or oxalate cannot be used, since they alter the osmotic pressure of the sample. For this purpose sinantrin in a 1:1000 dilution may be used. The use of this substance permits the taking of blood from the heart even at surrounding temperatures of 30 - 40°C (Ryk, 1939). At lower temperatures (10 - 12°C) the blood of some fish may be taken without any anticoagulant. Blood samples should therefore be taken in cold rooms (temperatures below freezing point should be avoided).

Blood may be taken from the heart, from the caudal artery or from the branchiostegal vessels. For this purpose glass cannulas (Fig. 1) with glass-tipped rubber tubing for drawing up blood by mouth may be used. The apparatus illustrated in Fig. 2 can also be used. If the fish is sufficiently large to permit the obtaining of several milliliters of blood, the cannula should be connected to a 10 - 50 ml glass reservoir. Siliconized glassware should be

used. The needle is inserted into the heart of the fish and the blood is withdrawn by suction (through the second tube).

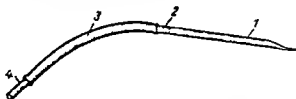


FIGURE 1. Cannula for obtaining blood samples from fish  
1-tapering end 2-cotton wool plug, 3-rubber tubing, 4-glass tip.

The cannula is prepared from low-melting point clean glass tubes. The wall of the cannula should be siliconized, and the capillary end of the cannula sealed by flame. The upper end is plugged with cotton (to prevent the inner part from being contaminated). Several cannulas must be prepared beforehand. Prior to use the sealed end is broken and a rubber bulb is fixed on the thick end.

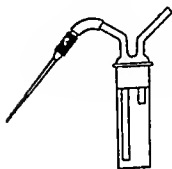


FIGURE 2. Apparatus for obtaining blood from fish

The rubber bulb is connected with the glass tip of the reservoir. For each blood sample, a fresh cannula should be used.

For studies on large fish, ordinary "Record" syringes may be used, although they are less convenient. After taking blood the syringes must be carefully rinsed with distilled water and alcohol.

For taking blood from the heart the fish should be dissected. The incision is made in the region between the pectoral fins (scissors or scalpel may be used). Having made a cut through the pericardium and exposed the heart, blood can be taken by puncturing the ventricle with a cannula or syringe.\*

Care should be taken that the blood is not mixed with even one drop of water. The site from which blood is to be taken (heart, gills, etc.) must be wiped dry with filter paper or hygroscopic cotton.

Blood samples are transferred into test tubes. The size of the test tube used depends on the amount of blood taken. Test tubes should be clean and siliconized. Test tubes filled with blood should be stoppered to prevent evaporation of plasma. The blood is then centrifuged to sediment blood cells and fibrin. Small test tubes may be stoppered with rubber corks or plugs. Blood should be centrifuged at 3-4,000 rpm for 15-20 minutes. Serum separated from the sediment of blood cells and fibrin is poured into the tube of the freezing-point apparatus. If the osmotic pressure is to be determined by means of another method (by tensimetry or microcryoscopy), serum is taken

\* For methods dealing with the obtaining of blood from the caudal vessels, see chapter on studies of fish blood.

directly from the centrifuge tubes. Until the determination of osmotic pressure the serum should be kept in a refrigerator (or in an ice box) to prevent bacterial growth. Our experiments showed that blood samples should be stored in the cold (at temperatures close to freezing point) for 48 hours. The freezing point of plasma does not change in the process. In this case the plasma should be sealed in ampoules (2-3 ml of plasma per ampoule). Ampoules are prepared from glass tubing. Fresh blood may be centrifuged directly in the ampoule. This circumstance enables one to take blood from fish in places which are far removed from the laboratory. Blood samples in ampoules may be stored and transported in vacuum flasks filled with ice.

#### CRYOSCOPIC (FREEZING POINT) METHOD

The freezing point of water containing dissolved substances is lower than the freezing point of pure water, i.e. the freezing point is below 0°C. Lowering of the freezing point is proportional to the amount of dissolved substance, and is not determined by the nature of the solute, but by the amount of solute particles dissolved in water.

Various nonelectrolytes in equimolar concentrations cause a similar lowering of freezing point. In the case of electrolytes a correction should be introduced - by multiplying the concentration of the solution by Van't Hoff's factor.

One gram-molecule of nonelectrolytes (glucose or sucrose) dissolved in water lowers the freezing point of the solution by 1.86°C. This value is called molar depression ( $\epsilon$ ). Lowering of freezing point is usually denoted by  $\Delta$ . According to Raoult's and Blagden's laws it can be concluded that

$$P = \epsilon \frac{m}{M} \quad (1)$$

where  $M$  - molecular weight of the dissolved substance and  $m$  - its concentration, expressed in grams per 1000 g of the solvent.

Having determined the freezing point of the solution studied when depression is ( $\Delta$ ), the total osmotic concentration of all solutes  $C$  can be calculated.

$$C = \frac{\Delta}{1.86} \quad (2)$$

The osmotic pressure of one molar solution of nonelectrolyte is 22.4 atm. at 0°C. If the freezing point of the solution (in degrees Centigrade) is known, the osmotic pressure of the solution can be calculated ( $P_{\text{osm}}$  expressed in atm.).

$$P_{\text{osm}} = \frac{\Delta}{1.86} \cdot 22.4 \quad (3)$$

Not infrequently, for the characterization of osmotic properties of biological fluids it is sufficient to determine the freezing point only. The freezing point of any solution may be determined with the aid of an accurate

mercury thermometer (Beckmann thermometer) or by means of the thermoelectric method. If the solution to be studied can be obtained in sufficient quantity (10 - 20 ml for each determination) the Beckmann method is the one of choice. This method is especially suitable for determination of the freezing point of the external medium (fresh, brackish or sea water and also of various salt solutions used in physiological experiments with fish). When only small amounts of biological fluids are available, as in studies on fry and small fish, one must resort to micromethods.

#### BECKMANN METHOD

Beckmann thermometer and Beckmann freezing-point apparatus are needed.

Beckmann thermometer (Fig. 3) has a large mercury bulb, a scale which has only six degrees and is graduated in  $\frac{1}{100}$  degrees and allows for readings with the accuracy to 0.002-0.003°C.

Scale readings are relative, i.e. the position of 0°C is arbitrary and not constant, 0° (zero point) may correspond to any temperature required (for example -5°C, -2°C, -10°C, etc.). For this purpose there is a small mercury reservoir at the top, into which mercury can be shaken if higher temperatures are used or from which mercury can be drawn (by warming the bulb until the mercury thread enters the reservoir) if lower temperatures are used.

For determination of the freezing point of biological fluids, mercury in the thermometer should be adjusted so that the freezing point of water (0°C) will be 3 - 4°C above the zero point of the scale. At first the thermometer is roughly adjusted, and then the exact position of freezing point of pure water is located on the scale.

**Rough adjustment.** The lower part of the thermometer with the mercury bulb is immersed in water with pure thawing ice or on snow and the position of the mercury column is recorded.

If the mercury stops somewhere in the middle of the scale, then the amount of mercury in the bulb does not have to be measured, only the exact position of the zero point remains to be determined.

If the mercury stops too high (above the upper edge of the scale) the amount of mercury in the bulb must be reduced. The mercury bulb is warmed by hand (or carefully on a burner) until the mercury enters the small reservoir. The upper end of the thermometer is shaken (by tapping with the finger) until the mercury thread is separated into two, and some of the mercury from the bulb enters the upper reservoir. The mercury bulb of the thermometer is again immersed in thawing ice and the position of freezing point is determined.

If the zero point is too low (below the middle of the scale) the bulb is again warmed until the mercury thread from the bulb enters the upper reservoir, and then the thermometer is turned upside down and some of the mercury is shaken from the top reservoir and into the bulb.

FIGURE 3.  
Beckmann  
thermometer



After the freezing point has been tentatively established, its accurate position is determined by using bidistilled water and the Beckmann freezing-point apparatus.

The Beckmann freezing-point apparatus (Fig. 4) consists of a vessel for the cooling mixture (outer jar) and a vessel for the solution to be studied, the latter is provided with an air-jacket. This apparatus can be constructed by the investigator himself. Cylindrical or rectangular glass beakers (1500 - 2000 ml volume) can be used as outer jars (for the cooling mixture).

The vessel for the sample solution (Fig. 5) consists of a wide tube (15 - 20 ml volume) with a side arm. The tube is provided with a cork stopper, through which a glass or ebonite stirrer and Beckmann thermometer pass.

The tube is fitted through a cork into an air jacket which ensures the uniform cooling of the vessel. In the lid of the apparatus there is another hole for a second stirrer to stir the cooling mixture.

The cooling mixture consists of three parts of crushed ice or snow and one part of sodium chloride. Fine table salt may be used. The temperature of the eutectic mixture approaches  $-21^{\circ}\text{C}$ .

Determination of zero point in Beckmann thermometer. Tube 1 (Fig. 5) is filled with 10 - 15 ml of bidistilled water and stoppered with the cork provided with the Beckmann thermometer and stirrer. The tube is fitted into the air jacket, and the entire apparatus is fitted into the lid of the Beckmann freezing-point apparatus. The vessel 3 (Fig. 4) is filled with the cooling mixture. The sample solution is continuously stirred and the position of mercury thread is watched. The temperature of beginning of freezing should be taken. Mercury falls gradually and then remains steady for some time. Readings are taken at the time when the mercury remains steady. In some instances supercooling may occur. In this case the mercury will fall below freezing point, then suddenly it will commence to rise and remain steady at the freezing point, which is then recorded. After some time the mercury commences to fall as a result of cooling of the ice formed.

The determination is repeated 3 - 4 times, each time with a fresh portion of water. The average value is calculated.

Freezing point of sample. The procedure is the same as that for determination of the freezing point of distilled water. Not fewer than two determinations should be made with each sample. The average value is determined and subtracted from the average value of the freezing point of bidistilled water which corresponds to  $0^{\circ}\text{C}$ .

Example.

#### Thermometer readings

##### 1. Freezing point of bidistilled water

Exp. 1	2.038°
Exp. 2.	2.095°
Exp 3	2.099°
<hr/>	
Average	2.097°

##### 2. Freezing point of the liquid studied (glucose solution)

Exp. 1.	0.920°
Exp 2.	0.918°
Exp 3.	0.917°
<hr/>	
Average	0.918°

$$\Delta \text{ of the solution} = 2.097^{\circ} - 0.918^{\circ} = 1.179^{\circ}$$

The Beckmann method is a classical one. It has been verified by numerous investigators and is widely used in chemistry for determination

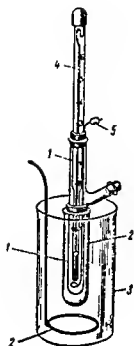


FIGURE 4 Beckmann freezing point apparatus

1 tube for the sample (solution examined); 2 air jacket; 3 outer jar for the cooling mixture; 4 Beckmann thermometer; 5 stirrer



FIGURE 5 Detail of Beckmann freezing point apparatus

1 tube for the sample (solution examined); 2 air jacket; 3 stirrer; 4 Beckmann thermometer; 5 side arm

of the molecular weight of inorganic and organic compounds. The experiments of T. N. Sumarokova and G. I. Aksenov (1958), however, showed that this method can be made even more accurate when the ordinary stirrer is replaced by an electric one which may stir the sample at any desired speed. This method allows for the rapid determination of the temperature of thawing at the moment of disappearance of the ice crystals.

**Veselov's microcryoscope.** We constructed a microcryoscope based on the principle of Drucker and Schreiner (1913) which enables one to carry out several simultaneous determinations on small samples (Veselov, 1936).

The apparatus (Fig. 6) consists of a microcryoscope, cooler, and electric stirrer (set in motion by an electric motor).

The main part of the microcryoscope is a glass cryostat for the gradual thawing of frozen samples. The cryostat consists of two cylindrical glass vessels fitted one into another. The outer vessel is about 12 cm in diameter and 20 cm in height. The inner vessel is fixed in the outer one on cork so

that the inner vessel is in no direct contact with the outer vessel. The vessels are covered with a wooden (or better with an ebonite) lid on which all other parts of the microcryoscope are fitted. The inner vessel is connected with the cooler by means of glass and rubber tubes. From the cooler  $\text{CaCl}_2$  solution is fed into the inner vessel. In the center of the lid there is a hole with a rubber plug for inserting the Beckmann thermometer. At one side of the lid there is another rubber plug through which an ordinary chemical thermometer with a scale to  $-10 - 15^\circ$  passes.

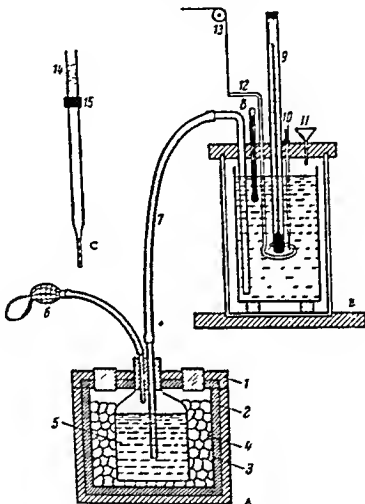


FIGURE 6. Apparatus for microdetermination of freezing point (After Veselov)

A-cooler, B-microcryoscope, C-glass tube with a capillary tip for samples, 1-wooden box, 2-felt insulator, 3-zinc box, 4-cooling mixture, 5-liquid medium for microcryoscope, 6-rubber bulb for pumping of air, 7-rubber tube, 8-thermometer for tentative determination of the temperature of the liquid medium, 9-Beckmann thermometer, 10-glass tube for samples, for the sake of clarity it has been drawn somewhat aside from the Beckmann thermometer. In reality the capillary part should touch the bulb of the thermometer; 11-funnel for pouring in of liquid medium, 12-stirrer, 13-connection between motor and stirrer, 14-cotton, 15-rubber ring to fix the tube on the lid of the microcryoscope

In the lid of the apparatus there are five holes arranged in a semicircle in relation to the Beckmann thermometer for the samples. The holes should be as close to the Beckmann thermometer as possible (the capillaries holding the samples should touch the thermometer bulb). In this way the possible difference between the temperature of the sample in the capillary and the reading of the Beckmann thermometer is diminished to a minimum.

In addition to the Beckmann thermometer, a chemical thermometer, capillaries for samples, and the stirrer, the lid is fitted with a funnel through which a liquid medium ( $\text{CaCl}_2$ ) at room temperature may be added to the cooled liquid. Since in the course of the determination the walls of the inner tube are covered with water vapor they should be wiped dry periodically with swabs, rubber or peoplist sponges, for the passage of which there is a special semicircular hole at the edge of the lid (4-5 cm long and about 1 cm wide).

As a cooling mixture 50% solution of calcium chloride should be used (instead of the glycerol recommended by Drucker and Schreiner). Calcium chloride solution does not freeze even on strong supercooling, and warms up more slowly than glycerol. Owing to this a higher degree of accuracy is achieved.

A ring-shaped glass or ebonite stirrer is used to ensure uniform change in temperature of the  $\text{CaCl}_2$  solution in the cryoscope. The stirrer is brought in motion by means of a small electric motor (up and down motion). The speed of the stirrer can be regulated by means of a rheostat.

The liquid medium (calcium chloride solution) is precooled in a cooler. The cooler consists of an outer wooden box, an inner metal box, and a 2000-2500 ml wide-necked glass bottle. The wooden box should have thick walls and a hermetically sealed lid. Its size is  $32 \times 22 \times 26$  cm. The lid is provided with two holes about 6 cm in diameter, closed by corks, and a third hole in the center of the lid for the neck of the glass bottle. The inner side of the bottom, the walls and the lid of the box are covered with a double layer of felt for insulation, inside the wooden box there is a lidless metal box made of zinc or zinc-coated tin plate.

The glass bottle with the cooler is placed in the zinc box so that its neck shows up through the hole of the cooler's lid. The stopper of the bottle is fitted with two glass tubes. One tube, the short one, is connected with a rubber bulb for pumping air, and the other tube reaches to the bottom of the bottle and is connected with the microcryoscope by means of a rubber tube. The tube is provided with a clamp. When air is pumped into the jar, the cooled solution passes from the jar and into the cryoscope.

Before the determination the zinc box is filled with cooling mixture (snow or crushed ice with sodium chloride).

Capillaries for holding samples are prepared from thin-walled glass tubes 0.3-0.5 cm in diameter (for this purpose a burner used by glass-blowers is used). A large reserve of capillaries should be prepared beforehand. Glass tubings for the preparation of capillaries should be carefully washed. To prevent the capillaries from getting dirty they should be plugged with cotton (at the wide end) and sealed (at the tapering end). The capillaries should be stored in closed boxes or glass jars with cotton on the bottom.

The sealed tip is broken off and the liquid (plasma, for example) is drawn in by suction from the test tube in which it had been stored. The height of the liquid (plasma) column should be about 0.5-1.0 cm. Then the capillary

tube is held with the tip up and shaken to break the liquid column into small columns, each 1.0 - 1.5 mm in length. The capillary tube is tipped up in order to propagate the liquid to the middle of the capillary. The tip of the capillary is then sealed on an alcohol burner (the sample in the tube should not become heated in the process).

The sample is then frozen in a cooling mixture consisting of snow or crushed ice covered with a thick layer of sodium chloride. The cooling mixture is placed in a large porcelain crucible or mortar, and ground until a slurry is formed. The temperature of the mixture drops to  $-19$  and  $-21^{\circ}\text{C}$ . To freeze the content of the capillaries they are plunged into this slurry for several seconds. Unless the cryoscope is ready, the frozen samples may be kept in the cooler, they are inserted through one of the side holes in the lid of the cooler.

The determination is carried out in the following manner. The bottle of the cooler is filled with 2,000 - 2,500 ml of filtered 50% calcium chloride solution. The cooler is filled with a cooling mixture consisting of snow or crushed ice and salt. The liquid medium ( $\text{CaCl}_2$  solution) is cooled for 10 - 20 min. The temperature of the liquid medium should be lower by at least  $1.5 - 2^{\circ}\text{C}$ , than the anticipated freezing point of the sample. While the liquid medium is being cooled, one may carry out other preparatory work (obtain blood samples from the experimental fish, centrifuge them, prepare capillaries with samples, etc.). When everything is ready an adequate amount of calcium chloride solution (cooling mixture) should be transferred from the cryostat into the cryoscope (freezing-point apparatus). Not less than  $3/4$  of the volume of the inner vessel should be filled with this solution. If the calcium chloride solution is inadequately cooled, it is again transferred into the cooler (by opening the clamp of the rubber tube which connects the microcryoscope with the cooler). \* If, on the contrary, the calcium chloride solution is too cold, it may be diluted with a solution at room temperature which is poured through the funnel fixed in the lid of the cryoscope.

When the temperature of calcium chloride solution reaches the desired value, the samples are frozen and the tubes with the samples are placed into the corresponding holes in the lid of the cryoscope. The stirrer is switched on (gentle stirring is recommended).

After some training 3 - 5 samples may be determined simultaneously.

A further task consists of watching the gradual thawing of frozen liquid columns of the sample and the position of mercury in the Beckmann thermometer. Since each capillary contains several columns of the sample, one of the middle columns should be watched. The columns on both extremities are unsuitable for this purpose, since the concentration of osmotically active substances there may be somewhat changed owing to evaporation during sealing of the tubes, etc. The smaller the column of liquid, the more suitable it is for the determination (the height of the column affects the accuracy of determination). The column of liquid which is watched should be located at the level of the middle part of the mercury bulb of the Beckmann thermometer. In that case the difference between the true temperature of the sample and the reading of the thermometer is minimal.

For the sake of convenience an electric lamp with reflector should be placed behind the cryoscope. To prevent warming up of the cooler by the lamp a rectangular vessel filled with water is placed between the lamp and

\* The cooler should be placed 20 - 50 cm below the microcryoscope

the cryoscope. One side of the vessel is wrapped in oil-paper which disperses light.

The thawing of the sample must be watched with the aid of a large magnifying glass (7-10 cm in diameter) with an adequate focal distance. Readings of the Beckmann thermometer are taken at the moment of disappearance of the last ice crystal in the sample. This temperature corresponds to the freezing point of the liquid. Readings are taken with an accuracy to 1/1000 part of a degree. Simultaneous determination of freezing point of five samples usually takes 30-45 min.

**Accuracy of the method and sources of errors.** The main causes of inaccuracies and errors are as follows

- 1) change in concentration of the liquid when the sample is taken, drying up of sample (during centrifugation, filling of capillaries) changes due to incomplete sealing of capillary tubes.
- 2) thick walls of capillaries, and poor thermoconductivity cause wide differences between the temperature of the sample inside the capillary and thermometer readings.
- 3) incorrect position of the columns of the sample in relation to the thermometer. Our experiments showed that if the column of the sample is located 1.5 cm below or above the central part of the bulb of the Beckmann thermometer, an error is introduced which may amount to  $\pm 0.25^\circ\text{C}$ .
- 4) Inaccurate technique (when the moment of disappearance of the last ice crystal is not accurately determined, incorrect reading of scale of Beckmann thermometer).
- 5) too large volume of the sample (too high column).

In the course of the determination, the mercury of the Beckmann thermometer rises slowly, owing to a gradual increase in the temperature of the liquid cooler. The temperature should be recorded immediately with the disappearance of the last ice crystal of the sample. An error can be introduced if the reading is not made immediately on disappearance of the last ice crystal, but somewhat later. Some training is required to take correct readings.

The volume of the sample is of great importance. Drucker and Schreiner had shown that the smaller the column of the sample, the more accurate the determination. Our experiments with columns of liquid of various height have shown that accurate results are obtained when the volume of sample column does not exceed 1 cubic millimeter, and its length does not exceed the diameter of the capillary tube.

Comparative determinations of  $\Delta$  of solutions of  $\text{CuSO}_4$ , glucose and blood plasma of bulls, carried out by means of the Beckmann method, and that described above showed that the difference in results did not exceed  $0.02^\circ\text{C}$  (provided all conditions mentioned above were observed). This accuracy is adequate for biological purposes.

Various modifications of our microcryoscopic method have been described (Blinov, 1948, Belyaev, 1951, 1957). These modifications did not include any new principle, nor did they change the construction of our apparatus

#### THEMOCALORIMETRIC CRYOSCOPY METHOD

This method was used for biological purposes for the first time by N. A. Maksimov (1913), in his studies on the resistance of plant tissues to frost.

This method later found wide application in studies on the resistance of insects to low temperatures (Kalabukhov, 1935, Kozhanehikov, 1937) and the resistance of fish to low temperatures (Shmidt, Platonov and Person, 1936).

**Principle of the method.** Freezing point of the sample is determined (with the aid of a thermoelectrical needle), from the difference in potentials between two cohesions of different metals. One cohesion (thermo-needle) is placed in the sample which is subjected to freezing, and the other is placed in the thermos with ice thawing in distilled water at  $0^{\circ}\text{C}$  (Fig. 7).

The apparatus consists of a cryoscope and a thermoelectric apparatus

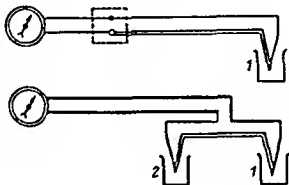


FIGURE 7. Diagram of thermoelectric apparatus (after Joffe, 1929)

1-sample with unknown temperature, 2-medium with accurately known temperature (thawing ice, for example)

**Cryoscope.** This part of the apparatus is based on the principle of the Beckmann apparatus. A 2,500 - 3,000 ml thick-walled cylindrical or rectangular jar provided with a wooden or ebonite lid may serve as an outer vessel (Fig. 8). Galvanized iron or tinned copper vessels may be used (Kozhanehikov, 1937). To maintain the necessary low temperature inside the inner vessel, the latter is placed in a wooden box lined with a thick layer of insulating material (sawdust, felt). The vessel is filled with a cooling mixture (ice or snow mixed with sodium chloride).

The main part of the cryoscope consists of two vessels fitted into one another. The outer vessel is double-walled. It serves as an air jacket. Between the walls of the inner and outer vessel there is a cryohydrate mixture, a thermometer and a ring-shaped stirrer. The inner tube is fitted with a small test tube 3 - 7 mm in diameter, which contains the sample and thermoelectric needle.

The double-walled vessel may be prepared from wide test tubes by placing one test tube inside the other with the aid of rubber rings or corks. In some cases the outer vessel may have a single wall. The stirrer is prepared from a glass or ebonite rod (if necessary, a thick tin or nickel-coated copper wire may be used as a stirrer). The stirrer is set in motion with the aid of an electric motor.

A 50% solution of calcium chloride may serve as a cryohydrate. A certain amount of calcium chloride should be precooled in a 1000 ml bottle by placing it in a vessel with a mixture of snow or crushed ice and sodium chloride. The cooled solution is poured into the cryoscope when needed.

The thermoelectric apparatus consists of a thermocouple, Dewar flask (may be replaced by an ordinary Thermos) and a galvanometer.

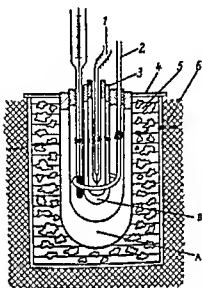


FIGURE 8 Diagram of thermoelectric cryoscope (after Maksimov, 1913, and Kozhanchikov, 1937)

A-vessel for calcium chloride solution (cryohydrate), 8-vessel for the sample; 1-thermocouple; 2-stirrer; 3-plug, 4-lid, 5-cooling mixture; 6-insulator

to the principle of the sight-vane (the slit in the galvanometer scale serves as a sight-vane). The accuracy of the reading is to 0.5 mm (the thickness of the thread pasted to the galvanometer glass).

**Thermocouple.** A correctly chosen thermocouple allows for the determination of temperatures to an accuracy to 1/100 of a degree. A constantan-copper thermocouple is preferred. It has an EMF of about 44 millivolts per 1°C, and its resistance depends only slightly on the temperature (Joffe, 1929). Nickel-copper thermocouples can also be used (Kalabukhov, 1935).

The general scheme of the connection between the thermocouple and galvanometer is illustrated in Fig. 7. The first joint (1) is placed in the sample whose freezing point is to be determined. The sample is placed

**Galvanometer.** The galvanometer should fulfill the following conditions: a) its sensitivity should be not less than  $15 \cdot 10^{-10}$  -  $25 \cdot 10^{-10}$  volts, b) its inner resistance should be low (8 - 30 ohms). A loop galvanometer is better than a mirror galvanometer. When a mirror galvanometer is used, the readings are made with the aid of a disk reflected by the galvanometer mirror. This method calls for work in dark rooms and for transformers or a small battery for mirror illumination. This drawback of the mirror galvanometer has been obviated by the ingenious device of N. L. Kalabukhov (1935). A thin black thread is pasted vertically on the glass window of the galvanometer. The scale is prepared from a compact white paper ruled into cm and mm with India ink. The figures on the scale should be drawn in the mirror reflection. The zero point of the scale is in the center; below it a small slit should be made. The scale is suspended against the galvanometer on two beams supported under its tripod at a distance of 50 cm from the galvanometer\*. The inscription on the scale should be turned towards the galvanometer (Fig. 9). Readings are made by watching, through the slit, the divisions and figures on the scale which are reflected in the galvanometer mirror. The readings are made according

to the principle of the sight-vane (the slit in the galvanometer scale serves as a sight-vane). The accuracy of the reading is to 0.5 mm (the thickness of the thread pasted to the galvanometer glass).

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\* The mirror galvanometer should be fixed on a main wall, since it is extremely sensitive to vibration



in the cryoscope tube. The second joint (2) is placed in distilled water with thawing ice (in a thermos or Dewar flask). Each joint with its copper end is connected with galvanometer contacts. With the difference in temperature between the first and second joints, a difference in potentials is formed in the circuit. The galvanometer shows the corresponding deflection, the magnitude of which is proportional to the difference in temperature between the joints.

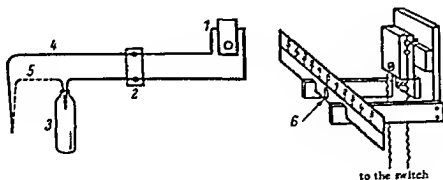


FIGURE 9. The use of a mirror galvanometer for thermoelectric cryoscopy (after Yalabukhov, 1935). On the left side - scheme of connections, on the right side - galvanometer and scale

1 - galvanometer; 2 - switch; 3 - thermocouple; 4 - copper wire, 5 - constantan wire, 6 - scale.

The wire for the thermocouples should be as thin as possible (from 0.05 to 0.1 mm). The ends of constantan and copper wires are fused or welded by tin. The joint should be as short and thin as possible (not more than 0.1 - 0.2 mm in diameter). Under these conditions the highest accuracy is obtained. The joint should be sharpened and again covered with a thin layer of tin. The needle should be periodically coated with a thin layer of a solution of rubber in benzene (to prevent corrosion by the various liquids tested - Maksimov, 1913).

The ends of the thermocouple should be fitted in a glass tip (Fig. 10). Two thin glass tubes are chosen so that one tube can be fitted into the other. The ends of the glass tubes should taper. The constantan wire passes through the capillary tip of the inner tube; the copper wire passes between the tubes. The thermojoint touches the capillary. The tubes are filled with paraffin. Wires protruding from the glass tip should be insulated and coated with rubber tubing. All wires should first be coated several times with an alcohol solution of shellac (for insulation).

A switch is placed between the thermocouple and the galvanometer, since it is necessary for switching the galvanometer on and off. In addition, with the aid of the switch one can work concurrently with several thermocouples, switching them alternately to the galvanometer. In selecting the type of the switch it is necessary to remember that no new sources of EMF should be created through contact between the various metals. Dickson's switch may be used (Fig. 11). This switch was recommended by Maksimov. It is convenient in that only copper wires of the thermocouple are in contact. For preparation of this switch a wooden test tube holder or a peg may be used. The insulating plate is prepared from a piece of ebonite or coverglass. For

better contact, the wire protruding from the end of the peg and to the plate should be flattened. The advantage of this switch over others is in that it switches on and off without introducing any other foreign metal which could create additional EMF.



FIGURE 10. Thermoneedle

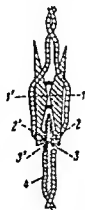


FIGURE 11. Simple switch

1-1'-leaves of wooden clamp; 2-2'-ends of galvanometer wire; 3 and 3'-ends of thermocouple wire; 4-insulating plate

N. I. Kalabukhov (1935) recommended to switch the galvanometer in circuit through an ordinary switch used in serials. One wire of the thermocouple and of the galvanometer is connected to one terminal, and the second wire of thermocouple and of galvanometer — to the second terminal. When these terminals are connected there is no current in the circuit. When the terminals are separated, current passes through the circuit.

The above simple scheme without additional EMF and resistance is possible because the scale is graduated when all resistances of the circuit are switched on.

Calibration of thermocouple. The coefficient of the thermocouple must be known, i.e. the number of divisions of the galvanometer (or the paper scale of the mirror galvanometer) which corresponds to 1°C. Calibration is done in the following way: The galvanometer is set at zero. Both thermojoins are immersed in water with ice in a Dewar flask. If the setup is assembled correctly, no deflection of the galvanometer needle should occur. Then the thermoneedle is immersed in another Dewar flask with water of different temperature, also determined accurately, for example 10°C. Deflection of the galvanometer needle is recorded, and the deflection of the galvanometer is calculated for one degree. The calibration curve should be constructed according to several points. Intermediate points are found by interpolation.

Calibration should be checked periodically. The work of the thermocouple should be frequently checked by comparing the results obtained with the thermocouple and with the Beckmann thermometer (or an ordinary exact mercury thermometer).

Determination of the freezing point is similar to that carried out by means of the Beckmann method, with the difference that instead of using the Beckmann thermometer, a thermocouple is used (put in the tube with the sample):

Procedure:

- 1) record galvanometer reading with open circuit.
- 2) place control and measuring thermoneedles into distilled water with thawing crushed ice, and check whether closure of circuit causes any deflection of the galvanometer needle
- 3) pour the sample into the tube of the freezing-point apparatus, immerse thermoneedle and place the tube in the freezing-point apparatus.
- 4) switch the galvanometer on
- 5) set the stirrer in motion and watch temperature changes (by galvanometer deflection). Record the reading corresponding to the freezing point.

Precautions. Accurate work yields reliable and accurate results. To avoid gross errors the following precautions should be observed:

- 1) temperature of terminals of each pair (of the switch and of galvanometer) should be identical. Terminals are wrapped in a thick layer of cotton
- 2) the position of the zero point of the galvanometer should be checked before and after the experiment.
- 3) one must check whether a difference in temperature is present in two segments of one and the same wire, since this may cause an additional current.
- 4) thick thermo-joints should be avoided, since they increase the error.
- 5) the work of the thermocouple should be checked by measuring the freezing point of nonelectrolyte solutions of known concentration, previously determined theoretically or by means of the Beckmann method (for example, the freezing point of an exactly 0.50 M solution of glucose).
- 6) the thermocouple coefficient should be periodically checked (by control determinations).

When all precautions are observed, this method permits the determination of the freezing point of 0.1 - 0.15 ml samples with an accuracy to 0.02°C.

#### TENSIMETRIC METHODS

The principle of tensimetry is based on the ability of dissolved substances to lower the vapor density of the solution. Vapor density is the pressure of vapor in equilibrium with the solution. Van't Hoff has theoretically shown that lowering of vapor density caused by solution of substances is proportional to its osmotic pressure. Lowering of vapor density by nonelectrolytes is proportional to the molar concentration of the solute. In the case of electrolytes, both molecular concentration and electrolytic dissociation play a part, since lowering of the vapor density is proportional to the total number of particles of the dissolved substance (molecules and ions) i.e. to the osmotic concentration.

The Barger (1924) - Raat (1928) tensimetric method for the determination of osmotic pressure of solutions is based on this principle.

The Barger-Rast method is suitable for field studies of fish physiology, since it does not call for complex equipment and the use of coolers, in addition, determinations can be carried out with small samples.

#### THE MODIFIED METHOD OF BARGER AND RAST (AFTER VESELOV)

The Barger-Rast method as modified by us is suitable for routine determinations of osmotic pressure of fish blood (Veselov, 1936).

**Principle.** A capillary tube is filled with a drop of the sample and a drop of NaCl solution of known concentration. Both drops should be separated by an air bubble. Both ends of the capillary tube are sealed, and the change in length of the column of both liquids is determined by means of a micrometer. A series of capillary tubes containing one drop of the sample and one drop of NaCl solutions of varying concentration is prepared. In the capillary tube with unchanged height of liquid columns (the length of liquid columns should be measured for several days) the NaCl solution is isotonic with the sample. This method enables one to detect differences in NaCl concentration to 0.01 M, in terms of osmotic pressure this constitutes about 0.5 atm.

**Capillaries.** Capillaries are prepared from low melting glass with the aid of benzine or alcohol glass-blowers. The length of the capillary tube is 7-8 cm, the inner diameter about 0.4-0.5 mm. The capillary tube should be of identical diameter along the entire tube length. A certain amount of training is required to prepare these capillaries. It is even better to use capillaries prepared by the method of B. V. Perfil'ev (1927), since they have an even and identical diameter throughout.

**Filling of capillaries.** One end of the capillary tube is filled with NaCl solution of known concentration (the height of the column should be 1-1.5 cm). The capillary end is sealed carefully to prevent heating of the solution. With similar precautions the middle part of the capillary tube is warmed, and the second end is rapidly filled with a) the sample (the height of the sample column 1.5-2.0 mm), b) air (height of air column 3-4 mm), c) another column of sodium chloride solution of similar concentration and d) air (the height of this column should be not less than 1 cm). The second end of the capillary tube is then sealed.

In the same way a series of capillaries are filled with the sample and NaCl solutions of varying concentration. The series of capillaries should be prepared in duplicates.

For convenience the capillaries are pasted to coverglasses with plasticine, several capillaries on each coverglass. The number of the capillary, the concentration of NaCl and the number of sample are marked near each capillary tube with a wax pencil.

**Measurement of the length of liquid columns.** When the series of capillaries (containing one sample) is prepared, the length of the columns of samples and that of the NaCl solutions is measured under the microscope with a micrometer (Fig. 12). The measurement is repeated after some time. If the sample is hypertonic in relation to the control NaCl solution, the column of the sample will lengthen; if the sample is hypotonic in relation to the control solution the column of the sample will shorten. The air bubble separating NaCl solution from the sample serves as a semipermeable

membrane. In the capillary in which no changes in the length of the liquid columns were detected, the sample is isotonic to the NaCl solution.

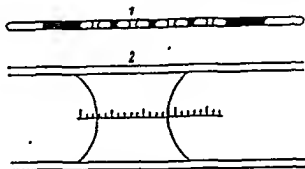


FIGURE 12. Tensimetric determination

1-sealed capillary tube with alternating columns of the sample and NaCl solution; 2-measurements of the length of the column of the sample (by microscope).

Final equilibrium, after which no changes in the length of columns are observed, is usually attained after 13 - 15 days. One does not have to wait 13 - 15 days; the second determination may be made 24 hours after beginning of the experiment. At this time the tendency of the sample column to lengthen or shorten is well pronounced.

**Checking.** Experiments to determine the optimal conditions for this method, as far as the accuracy and sensitivity are concerned (the experiments were conducted with blood plasma and sodium chloride solutions), showed that:

- 1) the length of the columns of sodium chloride solution and that of the sample should be about 1.5 - 2.0 mm.
- 2) the distance between the columns should not be less than 3 - 4 mm, otherwise both columns may merge.
- 3) the optimal inner diameter of the capillary tubes is about 0.03 mm.
- 4) at maximal differences in the concentration between plasma and NaCl, equilibrium may be attained in the course of 10 - 15 days.

5) by means of the tensimetric method one is able to detect differences between the control solution and the sample up to 0.0062 M, in practice it suffices that each control solution should differ from the next one by 0.0125 M.

In experiments with blood of fresh-water fish, 6 - 7 concentrations of NaCl are usually used (from 0.07 M to 0.15 M). Since the experiments are carried out in duplicates, 14 capillary tubes are required for each determination. Each determination takes 35 - 40 min.

The following possible sources of error should be eliminated beforehand: 1) uneven inner diameter of capillary tubes; 2) inaccurate filling of capillaries (especially careless heating) which may lead to a change in concentration of the control solution and the sample.

**Yastrebov's modification.** One of the drawbacks of the Barger-Rast method which detracts from its accuracy, is the invariable partial mixing of the control with the sample, owing to the fact that both liquids are introduced into the capillary tube through one and the same end. To

eliminate this drawback, M. T. Yastrebov (1954) recommended the introduction of one drop of NaCl solution (control) through one end, and one drop of sample through the other end, i. e. only two drops per capillary tube, one of the sample and one of the standard solution. For this purpose on the central part of the capillary tube a small capillary side-arm is formed by means of a microheater (Fig. 13). For the experiment the capillary tube is fixed in a frame (the author constructed a small holder) with the aid of two pieces of porcelain or glass tubing and two pieces of rubber sponge. During filling of the capillary, air comes out through the side-arm. The air bubble separating the sample from the control liquid should be 2-3 mm long. After the capillary tube is filled with the sample and the NaCl solution, all three openings of the capillary are sealed by flame (Fig. 14) and the capillary tube is pasted to the coverglass with wax. All other procedures are similar to those described above. The accuracy of determinations (according to M. T. Yastrebov) is up to  $10^{-7}$  M NaCl.

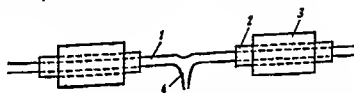


FIGURE 13. Preparation of capillary tube with side-arm for tensimetry (after Yastrebov, 1954)

1-capillary; 2-a piece of porcelain or glass (to fix the capillary) 3-a piece of rubber sponge; 4-arm

Hill (1928, 1930) proposed that the change in temperature caused by evaporation of the liquid on the surface of a very sensitive thermoelement should be used for determination of the tension of vapors of aqueous solutions. This method is also suitable for the determination of osmotic pressure of solutions, since lowering of vapor tension caused by dissolved substances is proportional to the osmotic pressure of the solution. Since this method has not found wide application in studies on fish and is described in the work of Veselov (1959), it will not be described here. For the same reason we shall not dwell on osmometric and plasmometric methods.



FIGURE 14. Sealed capillary tube with side-arm prepared for tensimetric determination of osmotic pressure (after Yastrebov, 1954)

1-sample, 2-control solution; 3 side-arm

DETERMINATION OF OSMOTIC RESISTANCE OF ERYTHROCYTES  
(FRAGILITY TEST)

The resistance of erythrocytes to hypotonic solutions differs in different fish species. It may also change under the influence of various internal and external factors, especially during more difficult life conditions or additional factors such as infectious and parasitic diseases. Hence studies on osmotic resistance of erythrocytes as one criterion for characterization of the physiological state of the fish is of great interest.

**Principle.** When erythrocytes are placed in a hypotonic medium (distilled water or hypotonic sodium chloride solution), hemolysis takes place. Under the influence of osmotic forces erythrocytes swell, their membranes disrupt, and the blood is laked. The osmotic resistance of erythrocytes is expressed by the lowest concentration of NaCl which does not cause hemolysis.

**Procedure.** Twelve test tubes are filled with 2.5 ml of chemically pure NaCl solutions of varying concentrations. The usual range of concentrations is from 0.30 to 0.74% NaCl (for an example, see the following table):

Test tube, number	Concentration, %	Test tube, number	Concentration, %
1	0.30	7	0.54
2	0.34	8	0.58
3	0.38	9	0.62
4	0.42	10	0.66
5	0.46	11	0.70
6	0.50	12	0.74

The solutions are prepared by diluting an exact 10% solution of NaCl in distilled water. For example, to obtain 0.30% solution a 100 ml volumetric flask should be filled with 3.0 ml of the 10% NaCl solution and the volume is made up with distilled water. When the test tubes are filled with NaCl solutions of varying concentration, 0.05 ml of blood are added to each test tube with a micropipet. The mixture is shaken and left for one hour. Hemolysis is determined by comparing the test tubes.

Maximal osmotic resistance of the erythrocytes is expressed by the maximal NaCl concentration in the presence of which a small part of blood remained nonhemolytic, while in all test tubes to the left a complete hemolysis has taken place. On the right hand side of the series, a test tube with first traces of hemolysis is noted, while to the right of this test tube no hemolysis has occurred. The concentration of the solution in this test tube shows minimal osmotic resistance of erythrocytes.

METHODS OF STUDY OF THE FUNCTION OF SOME OSMOREGULATORY  
ORGANS

Osmoregulatory processes consist of at least two elements: regulation of water balance of the organism, and regulation of the mineral composition of the internal medium. These processes are accomplished in aquatic organisms with the participation of numerous organs: skin, respiratory organs (branchiostegal apparatus) excretory organs, and digestive tract.

\* [Presumably 0.58 is intended.]

Methods for collection of urine and quantitative estimation of renal function are dealt with in the next chapter by A.G. Ginetinski, V. F. Vasil'eva, M.G. Zaks, Yu. V. Natchin and M.M. Sokolova.

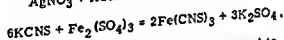
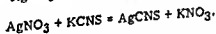
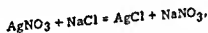
#### DETERMINATION OF CHLORIDES IN THE URINE BY THE METHOD OF VOLGAR

**Principle.** Urinary chlorides are precipitated by a known excess of a standard titrated solution of silver nitrate in the presence of nitric acid. The sample is filtered off and silver nitrate excess is titrated with potassium thiocyanate or ammonium thiocyanate in the presence of iron oxide as an indicator.

**Reagents.** For chloride determination the following reagents are necessary: 1) silver nitrate solution: 29.075 g of  $\text{AgNO}_3$  in 1000 ml of distilled water, 2) potassium thiocyanate solution 8.0 g of  $\text{KCNS}$  in 1000 ml of distilled water, 3) chloride-free nitric acid, 4) saturated solution of  $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ .

**Procedure** - 1) pipet 10 ml of urine into a 100 ml volumetric flask, 2) add (with pipet) 4.0 ml of nitric acid, 3) add (from buret) 20.0 ml of  $\text{AgNO}_3$  solution, 4) add water to the 100 ml, 5) filter the solution through a dry filter paper into a beaker, 6) transfer 50 ml of the filtrate into another beaker and titrate with potassium thiocyanate till the appearance of a red color (a ml of thiocyanate), 7) pour 10.0 ml of silver nitrate solution into another dry beaker and add 5.0 ml of nitric acid, 5.0 ml of  $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ , 80 ml of distilled water, and titrate with potassium thiocyanate (x ml of thiocyanate).

**Reaction:**



**Calculations:** Since x ml of thiocyanate correspond to 10 ml of  $\text{AgNO}_3$ , 2a ml are equivalent to  $\frac{10 \times 2a}{x}$  of  $\text{AgNO}_3$  solution. Hence  $20 - \frac{10 \times 2a}{x}$  is equal to the amount of silver nitrate (in ml) used up for the precipitation of chlorides from the 10 ml solution. We know that 1 ml of our solution of  $\text{AgNO}_3$  precipitates 0.01 g of  $\text{NaCl}$ , hence:

$$(20 - \frac{20a}{x}) \times 0.01 = 0.2 \times \frac{0.2}{x}.$$

Multiplying the result obtained by 10, the content of chlorides in urine (in percent) is calculated. This can be directly calculated by means of the formula

$$2 - \frac{2a}{x}.$$

It is enough to determine the value of x once for all analyses carried out with the given reagents.



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## METHODS OF STUDY OF OSMOREGULATING SYSTEMS IN FISH

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The assessment of function of the main organs of regulation of water-salt balance (kidneys, gills) as well as the determination of total concentration of osmotically active substances in blood is of great interest in studies on osmotic regulation in fish. The primary role of the kidneys, especially in fresh-water fish, consists in excretion of water excess, while the excretion of metabolic wastes is of secondary importance. In contrast to mammals, kidneys of fresh-water fish function as an organ concerned with maximal preservation of sodium salts and not with their excretion. The real osmotic regulation is extrarenal. For this purpose special cells are present in the gills, and sometimes in other parts of the body surface, which excrete sodium or take up sodium from the external medium in sodium deficiency.

Assay of renal function is done by methods which allow for quantitative analysis of diuresis, glomerular filtration, and active excretion. As to the gills, there are certain methods for assaying the functional state of the structures involved in sodium transport.

It is not our task to give here a comprehensive review and description of all the methods used in studies on osmotic regulation in fish. We only wish to share our experience in this field, and to describe the methods used by us in the form in which they are used in practice.

### DETERMINATION OF OSMOLAR CONCENTRATION OF BIOLOGICAL FLUIDS AND MEDIA WITH THE AID OF CRYOSCOPY

It is known that the freezing point of any solution is lower than that of the pure solvent. The difference in temperature between the two freezing points (or depression  $\Delta^*$ ) allows one to determine the total molecular and ionic concentration of the dissolved substances.

For determination of the freezing point, an apparatus provided with a resistance thermometer (thermistor), serving in the capacity of one of the shoulders and switched into the scheme of an ordinary electric bridge, is very convenient. Into one diagonal of the bridge a galvanometer is connected, and a current source into the other (Fig. 1). The bridge is balanced at

0°C. Changes in temperature in either direction cause changes in the resistance of the thermistor, disturbing the equilibrium of the bridge, a phenomenon which forms the principle of the method.

Thermistors of the MT-54 type with a resistance of 4000 - 7000 ohms at 0°C are used. Such thermistors are produced by the Institute of Agrophysics in Leningrad. Two thin wires enclosed in a short glass tube are welded to a very small spherical semiconductor. Their ends protrude outside for connection with flexible insulated wires which serve for switching them into the scheme. The entire construction is enclosed in plexiglas (non-metal!) case, so that only the semiconductor remains outside and all the remaining parts are securely insulated (Fig. 2) The semiconducting material from which the thermistor is constructed has an extremely high temperature coefficient, due to which any changes in temperature cause marked changes in the resistance of the thermistor.

FIGURE 1 Diagram of electric bridge with resistance thermometer ( $R_t$ )

$R_1$  and  $R_2$ —constant resistors  
 $R_3$ —variable resistance  $R_4$ —shunt;  
 1—galvanometer, 2—battery, 3—key

of determinations) may be used. In the work with GES-47, an alternate shunt is connected parallel to the galvanometer, it allows for alteration of the sensitivity of the galvanometer which should be lowered in studies on concentrated solutions. When the shunt is changed, the value of each scale division is altered. Preliminary experiments should therefore be carried out to determine the value of each scale division with each of the shunts used

Under laboratory conditions accumulators serve as a source of power, in field conditions dry elements are used. Voltage of batteries is determined by the properties of the bridge. Various decimal bridges may be used for measurements. In our laboratory the shoulders of the bridge were chosen, so that the ratio between constant shoulders should be 1:10, and the alternating resistance should be 10 times higher than that of the thermistor. For the portable M-91-A galvanometer the universal UMV type bridge is used, the proper galvanometer bridge is disconnected in the process, and the ratio between the shoulders is 1.1 when the thermistor's resistance is 4000 - 7000 ohms. Such a bridge may be fed with as little as 1.5 volts

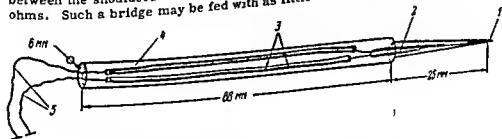


FIGURE 2. Thermistor  
 1—semiconducting sphere 2, 3 and 5—wires 4—plexiglas case of thermistor

The freezing point is determined in a special glass vessel with a double bottom (Fig 3), this form of vessel precludes the possibility of too rapid freezing of the sample. The vessel is cooled in a 200 - 500 ml glass jar, filled with cooling mixture (ice with salt) in such proportion that the temperature of the mixture should range between  $-5$  and  $-7^{\circ}\text{C}$ . If the jar is placed in a jacket with asbestos insulation, one charge with cooling mixture suffices for 10 - 15 determinations carried out in duplicates. The jar should be covered with a metal lid provided with a round hole for fixing the vessel. The lid is also provided with a tripod and clamp for fixing the thermistor (Fig. 4) in working position. If ice is not available, ether may be used instead. To cool the vessel the latter is wrapped in a layer of cotton wool and gauze soaking in ether.

Ether evaporates and cools the vessel. The rate of evaporation may be accelerated by blowing air on the vessel. The use of ether for temperatures below  $-15$  -  $-16^{\circ}\text{C}$  is impractical.

The first stage of the work consists in balancing the bridge at  $0^{\circ}\text{C}$ . Equilibrium will be attained when the product of resistances of the opposite shoulders are equal.

$$\frac{R_t}{R_3} = \frac{R_1}{R_2} \text{ hence } R_t = R_3 \frac{R_1}{R_2}$$

Since the values of  $R_1$ ,  $R_2$  and  $R_3$  are known, the value  $R_t$ , i. e. resistance of thermistor at  $0^{\circ}\text{C}$ , may be calculated.

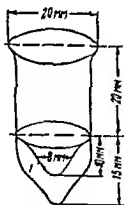


FIGURE 3 Vessel for freezing  
1-double bottom

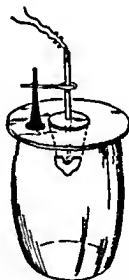


FIGURE 4 General view of apparatus for freezing (samples)

In practice this is done as follows. First the resistance of the thermistor is roughly determined by immersing it in distilled water precooled to  $0^{\circ}\text{C}$ .

For this purpose the jar is filled with thawing ice (not with cooling mixture), and the vessel is filled with one or two drops of distilled water in which the tip of the thermistor is immersed. This should be done carefully so as not to damage its working part. The thermistor is fixed in a tripod with a clamp. Through changing alternating resistance the thermistor is prepared so that the galvanometer needle should show no passage of current. The zero point of the apparatus is finally determined. For this purpose, resistance on the alternating shoulder  $R_3$  is adjusted approximately to that of the thermistor  $R_t$ . The vessel with distilled water and the thermistor is placed in a cooling mixture. At first the pointer of the galvanometer is deflected towards the "warm" side (Fig. 5). With decreasing temperature of the water it will move towards zero, and on supercooling, it will move towards the "cold" side. At this moment a small crystal of ice scraped from the external surface of the jar filled with cooling mixture is introduced into the water by means of a thin glass rod.\* This causes a rapid freezing of the supercooled water - the pointer will move back and will stop at a point corresponding to the freezing point of the distilled water, i.e. the reading on the scale will correspond to  $0^\circ\text{C}$ . If the galvanometer shows a passage of current, the alternating resistance  $R_3$  should be changed and the galvanometer pointer set at the zero of the scale, i.e. the bridge is balanced. The determination is repeated three times, and the average value of  $R_3$  is then determined, this is left unchanged. The apparatus is then ready for use.

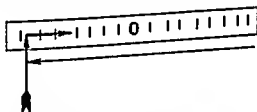


FIGURE 5 Movement of galvanometer pointer along the scale during determination of freezing point

To the right of zero point - "warm" side, to the left, "cold" side. Arrow below the scale indicates the movement of the pointer during cooling and supercooling of the sample; arrow on the left, perpendicular to the scale, shows the position of the pointer at the moment of introduction of the ice crystal; the arrow on the scale indicates the movement of the pointer at the moment of reading of the sample

The next stage consists of graduating the resistance thermometer. For this purpose an exact 1% solution of chemically pure NaCl is prepared its freezing point is equal to  $-0.60^\circ\text{C}$ . The jar is fitted with cooling mixture, a well-dried vessel is filled with 1-2 drops of the solution, the thermistor is immersed in the solution, and the freezing point of the latter is determined as described above. On supercooling, a small ice crystal is introduced

\* When ether is used ice crystals are taken from the gauze cover

The solution freezes. Owing to evolution of latent heat of thawing, the pointer will move towards the "warm" side and will stop indicating the termination of this process. Scale readings are converted into degrees centigrade in the following way.

If freezing of a 1% solution of sodium chloride causes a deflection of the galvanometer by  $h$  divisions from the zero point, then each division is equal to  $\frac{-0.60}{h}$  degrees Centigrade.

Determinations with standard solutions should be repeated several times, and the average value is taken. Each determination of the unknown sample should be followed by checking the graduation scale with the aid of 1% solution of NaCl as described above (to prevent the possibility of chance alterations in electric characteristics of the circuit).

For determination of the osmolar concentration of samples, the same procedure is carried out. The freezing point ( $\Delta^*$ ) is calculated by multiplying the reading of the scale by  $\frac{-0.60}{h}$  (factor = degree/scale division). Each determination should be carried out in triplicate, the average value is taken. The final result is expressed in milliosmoles, i.e. units of concentration of osmotically active substances equivalent to 0.001 M solution of nonelectrolyte. To translate the value characterizing  $\Delta^*$  into milliosmoles, the freezing point

in degrees should be multiplied by the coefficient  $\frac{1000}{1.86}$  i.e. by 537.

Determination of the freezing point with the aid of a thermometer has a number of advantages over the Beckmann method and its modifications, and also over the methods making use of thermocouples. The small weight of the working part of the thermometer and the insignificant heat inertia allows for determination of freezing points with an accuracy to 0.015 - 0.003°C, using 0.03 - 0.05 ml samples. Each determination takes 3 - 4 minutes. The productivity and convenience of this method are such as to make all previous methods obsolete and impractical.

**Obtaining of blood.** The simplest method is as follows. The fish is held vertically. The caudal peduncle is severed obliquely at its base, and blood dripping from the cut is collected in a test tube.

**Urine collection.** Depending on the aim of the study, urine from free-swimming fish may be collected by various methods. The simplest (not requiring special equipment) is that based on the use of the volume capacity of the urinary bladder of the fish itself. The fish is taken out of water and urine present in the urinary bladder of the fish is squeezed by gentle massaging of its abdomen. The external urogenital opening is then sewn with a purse-string or cross-like suture, and the fish is returned to the aquarium. From this moment urine is collected. The time needed for urine collection depends on the size of the individual fish, fish species and experimental conditions. In fresh-water fish under natural conditions the diuresis is 0.3 - 0.4 ml per 100 g body weight per hour. In such cases the urine should be collected after 1.5 - 2 hours, in the case of marine fish whose diuresis is smaller, amounting to 0.1 ml of urine per 100 g body weight per hour, urine should be collected after 4 - 5 hours and sometimes more. After this time the fish is taken out of the water, its abdomen is incised, and urine is collected by means of a syringe or through a cut made on the apex of the urinary bladder.

This method permits the use of one fish only once. If experimental conditions call for a repeated collection of urine from one and the same specimen, thin catheters ending in small bulbs may be used. Such bulbs are prepared from thin rubber and polyethylene or polyvinyl chloride tubes of suitable diameter.

As in the first case, prior to the experiment urine in the urinary bladder should be removed and the edges of the urogenital opening with the catheter inside should be tightened by means of a purse-string suture. Before introducing the catheter, the position of the urinary bladder of the fish should be ascertained, in the different fish species the latter may be directed either cranially or caudally. The catheter should not be pushed too far, so as not to damage the urinary bladder. The bulb should be tightened with an additional suture at the base of the bladder. Before introducing the pouch or bulb, the air from the latter should be squeezed out.

The bulb may be prepared in such a manner that new bulbs may be changed without removing the catheter. For this purpose the catheter is connected with the sac by means of a small tube.

In the case of rays, in which the ureter opens into the cloaca, the cannula with the rubber bulb should be sewn above the outlet of the ureters (where a pocket-like fold is present in the cloaca). In this case the cannula should consist of a tube with edges for tightening the purse-string suture, resembling a small gastric fistula.

Finally, in some cases where it is necessary to follow the development of diuresis, urine may be collected continuously. This method calls for a special aquarium with an opening in its floor. The opening is fitted with a stopper through which a tube is inserted. The catheter placed in the urogenital opening of the fish is connected with a thin elastic tube which, in its turn, is connected with the tube passing through the stopper. Urine passes through the catheter and the various tubes and into a micropipet or some other measuring device placed under the opening in the floor of the aquarium. The entire system should be sealed hermetically and filled with liquid. Movements of the fish are restricted by placing a net along both sides of the animal. The aquarium should be aerated or provided with running water. For such experiments large fish weighing at least 500 g should be used (Fig. 6).

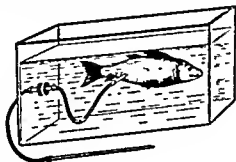


FIGURE 6 Continuous collection of urine



## QUANTITATIVE ASSAY OF RENAL FUNCTION

Methods for quantitative assay of renal function are based on the corresponding analyses of blood and urine. To determine renal function in fish the same tests used in warmblooded animals are employed. Inulin is injected when the extent of filtration and reabsorption is to be determined. Paraamino-hippuric acid and diodrast are administered if secretory activity or renal blood circulation are to be determined. These compounds are injected intramuscularly 1.5 - 2 hours before the experiment; Doses per 100 g body weight: inulin, 50 mg; paraamino-hippuric acid - 0.03 ml of 20% solution; diodrast, 0.02 ml of 35% solution. For determination of the excretory function of the renal tubuli, the dose of paraamino-hippuric acid and that of diodrast should be increased 10 to 15-fold. For the theoretical basis of these tests, see the work of Ginetsinskii\* which also contains references on the biochemical methods for determination of various substances in the urine.

For quantitative tests, blood is first collected and then the abdominal cavity is opened and the amount of urine determined.

## STORING SAMPLES IN AMPOULES

Various biological fluids and media (blood, urine, water from natural water reservoirs or experimental media) may be stored in ampoules until analyzed. This is done when the analyses must be done in special, well-equipped laboratories (for example, analyses by means of flame photometry). Normally 3.0 to 20.0 ml ampoules are used. The ampoules should be washed in the same way as all chemical glassware (treatment with chromate mixture, rinsing in tap water and distilled water, and drying in desiccator).

The ampoule is filled with the sample to not more than  $\frac{3}{4}$  of its volume, and sealed. The sample is introduced into the ampoule by means of a Pasteur pipet. Small ampoules from low-melting glass may be sealed over the flame of an ordinary alcohol burner, those prepared from high melting glass require a hotter flame. After some training one can learn to seal such ampoules on the flame of a primus-stove.

Sealed ampoules are wrapped in gauze and boiled in a sterilizer for one hour. If many ampoules are sterilized together, they must be marked. For this purpose numbered aluminum rings placed on the necks of the ampoules may be used.

After sterilization the ampoules are marked by labelling them. The inscription on the label may be done with a simple pencil.

Preservation of liquids with high protein content which coagulates during sterilization presents certain difficulties. However, if the sample is to be analyzed for ionic content by means of flame photometry, it can first be centrifuged, the coagulated protein discarded, and the supernatant fluid examined.

## STUDIES ON GILL CELLS INVOLVED IN SODIUM TRANSPORTATION

For an assay of the functional state of the system involved in sodium transportation, Case-Wiimer cells should be studied. These cells are usually

\* Collection "Physiological Methods in Clinical Practice" Medgiz, p. 139, 1959.

located at the base of the branchiostegal leaves or between them (Fig 7). They can be easily differentiated from cells of the respiratory epithelium of the gills by their height and also by the characteristic eosinophilic granulation of their cytoplasm.

For histological studies, a small segment of the arch (2 - 4 leaves) is taken. The material is immediately placed in Bouin's fixing fluid (saturated aqueous solution of picric acid, 15 ml, formaldehyde, 5 ml, and glacial acetic acid, 1 ml, mix immediately before use). Fixation should last from 2 to 24 hours, depending on the size of the sample. Longer fixation does no damage to the tissue. After fixation the tissue is transferred into 70% ethanol which should be changed two-three times. The preparation is embedded in paraffin by the usual histological technique. During embedding, the plane of the slice should be perpendicular to the longitudinal axis of the leaf, if possible. Staining with hematoxylin-eosin yields good results.

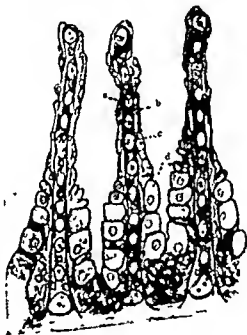
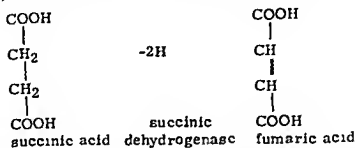


FIGURE 7 Case-Wilmer cells (d) in fish gills  
a-respiratory epithelium, b-capillary, c-erythrocyte

Increased sodium transportation is accompanied by an increase in the number of cells, and these can sometimes be encountered along the stem of the branchiostegal leaves.

For characterization of the enzymatic systems participating in sodium transportation in fish gills, we have been using a histochemical reaction for succinic dehydrogenase. This enzyme has been chosen because its activity changes depending on the intensity of sodium absorption or excretion, thus characterizing the state of energy-yielding systems involved in the sodium transportation.

Succinic dehydrogenase is one of the enzymes of the Krebs cycle which plays an important role in aerobic oxidation of carbohydrates. The function of succinic dehydrogenase involves the oxidation of succinic acid by removal of two hydrogens, and its conversion into fumaric acid.



**Assay of succinic dehydrogenase.** To the medium containing the gilia, an electron acceptor is added (for example various tetrazolium compounds) which is reduced by hydrogens removed from succinic acid by succinic dehydrogenase. Tetrazolium is a colorless water-soluble compound which in the reduced state (formazan), becomes colored and water insoluble.

Tetrazolium salts are reduced at various sites of the electron transport chain of succinoxidase, and this reaction characterizes not only the activity of succinic dehydrogenase, but also the functional state of the entire succinoxidase system.

The histochemical reaction carried out by the method of Shelton and Schneider yields good results in total preparations of branchiostegal threads without preliminary preparation of frozen allices in a microtome. To prevent movements of the fish, the apical cord is sectioned and the gill arch is quickly excised. The arch should be excised carefully so as not to damage the branchiostegal leaves. It is immediately transferred into a thermostat with the incubation reaction mixture. For the experiments, small fish weighing to 100 g should be chosen, if possible. The histochemical reaction reveals that all cell structures possess a considerable succinic dehydrogenase activity.

The incubation mixture contains equal volumes of the following solutions: 0.1 M phosphate buffer, pH 7.5, 0.1 % neotetrazolium dissolved in boiled distilled water, 0.1 M sodium succinate and boiled distilled water. For the preparation of 0.1 M phosphate buffer, 16 ml of 0.1 M monosodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) are mixed with 84 ml of 0.1 M disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ). 0.1 M solution of sodium succinate may be prepared from sodium succinate or from a commercial preparation of succinic acid which must be neutralized with sodium bicarbonate. For this purpose, 1.18 g of succinic acid (molecular weight 118) are dissolved in 100 ml of distilled water, and  $\text{NaHCO}_3$  is slowly added to a pH 7.0. It must be mentioned that clear-cut results are obtained only when freshly prepared solutions are used and the pH of the buffer is carefully adjusted. pH may be determined with the aid of pH indicators. Succinic acid solution should be freshly prepared each week and stored in a refrigerator. Buffer solution may be prepared once in 10-12 days.

The histochemical reaction should be carried out in a thermostat at 37° C under anaerobic conditions. 1.5-2.5 ml containers or dishes with a ground upper edge are filled with the incubation mixture, the tissue is placed in the mixture, and the vessel is covered with a lid. Hence the reaction proceeds without air access.

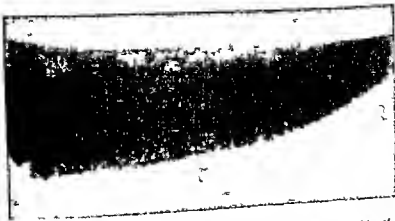


FIGURE 8. Succinic dehydrogenase activity in gills of Pacific mussels. Deposition of diformazan in the cells of gill leaves involved in sodium excretion

After 60 - 90 minutes of incubation the solution is carefully decanted and the gills rinsed with water, so that gill cells should not be damaged. After fixation in 5 - 10% neutral formalin for several hours the gills are again rinsed with water, the gill leaves are separated from the arch under a magnifying glass by means of a scalpel and embedded in warmed glycerol gel (15 g gelatin, 85 ml glycerol and 100 ml of distilled water).

In sites with high succinic dehydrogenase activity in the gill cells intense deposition of water-insoluble red-violet formazan can be seen. Enzymatic activity cannot be detected in all cells, but only in Case Wilmer cells which participate in sodium excretion. When all conditions are observed it can be seen that the entire succinic dehydrogenase activity as judged by formazan deposition is localized in the cell mitochondria (Fig 8).

## DETERMINATION OF FREEZING POINT OF BLOOD SERUM BY MEANS OF A MICROELECTROTHERMOMETER

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The osmotic pressure of any solution is determined by the concentration of molecules and ions of dissolved substances. Blood plasma of bony fish contains 2.5 to 7.0% of proteins and 1.2 to 1.8% of various salts. In spite of the high protein content, the osmotic pressure of blood plasma depends mainly on its salt content. Separation of plasma proteins from blood plasma affects its osmotic pressure only little. This is explained by the high molecular weight of proteins as compared to that of inorganic salts, which causes the amount of protein molecules in blood plasma to be negligible as compared to that of inorganic salts. Osmotic pressure is determined by the number of molecules in the solution and their dissociation. The higher the dissociation the higher the osmotic pressure. Changes in osmotic pressure of fish blood plasma is due mainly to changes in plasma salt concentration.

The osmotic pressure may be determined from the freezing point of the solution. The cryoscopy method is used for determination of the freezing point. Freezing point of pure water is at 0°C. If water contains dissolved substances, its freezing point will be lowered. Lowering of the freezing point in relation to that of pure water is called depression and is denoted by  $\Delta$ .

Methods available for determination of the freezing point of plasma are very complex, and in the majority of cases subjective. This greatly reduces their accuracy and handicaps the studies on osmotic pressure and osmotic regulation in aquatic animals. The laboratory of fish physiology of GosNIORKh has been engaged in studies on  $\Delta$  of fish blood plasma by means of a micro-electrothermometer. The microelectrothermometer is a portable electrical instrument designed for rapid measurements of temperature within an interval from -2.5 to +0.5°C. The thermometer transducer is a point-contact type, employing miniature thermal resistors whose resistances vary with temperature. The diagram (Fig. 1) shows a four-arm constant-current bridge. In one arm of the bridge transducer I or II is connected, the components in the other arms being always included. Bridge arm 6-9 is varied and designed to operate over several temperature ranges. The variable resistor II is used to provide an operating current before measurement over the 123 divisions of the scale. The instrument is supplied by a 4.5 volt flashlight battery located at the bottom of the instrument case. The entire range of the scale is 3°C and each division is 0.025°C, the minimum possible reading. A half division, 0.012°, may be determined visually. The scale has

been developed in the laboratory of fish physiology at the GosNIORKh The microelectrometer has been turned out by the experimental workshops of the Leningrad Institute for Postgraduate Studies for Physicians im. S M Kirov

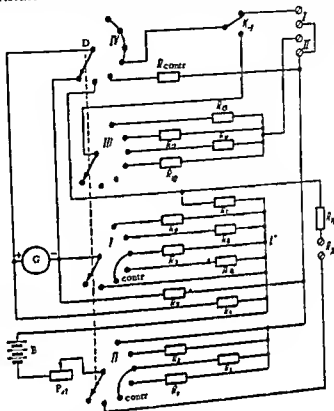


FIGURE 1 Microelectrothermometer diagram

$P_1$ -potentiometer, 10,000-20 000 ohms  $K_1$ -single pole toggle switch in position 2, B-battery, 4.5 volt G-electromagnetic galvanometer 15-20 microampere, D-range switch I-II-sockets for switching in transducers  $R_4$ - $R_5$ -resistors, 1000-5000 ohm (adjusted during calibration);  $R_6$ - $R_9$ -resistors, 1000 ohm,  $R_{10}$ - $R_{13}$ -resistors 500 to 5000 ohm (adjusted during calibration);  $R_{10}$ - $R_{13}$ -resistors 2-100 ohm (adjusted during calibration);  $R_{14}$ -resistor, 43,000-51,000 ohm,  $R_{contr}$ -resistor, 1500-2000 ohm;  $R_1$ -resistor of about 800 ohm (adjusted during calibration);  $R_X$ -connecting socket

#### PROCEDURE

Prior to operation the microelectrothermometer must be calibrated. First, the cover of the instrument (Fig. 2) is opened and the transducer is connected to the proper plug (Fig. 3), the plug number is engraved on the probe of the transducer. The switch on the right side of the upper panel (Fig. 3) should be in "arrest" position. The center switch is then turned to the side of the socket in which the transducer is inserted. With the aid of a screw, the scale pointer is set to the red line at the left side of the scale opposite the figure 2.5 The switch on the right side is set in "control"

position, and the "current adjustment" switch on the left side of the panel is used to adjust the pointer. The red line at the right side of the dial is set on the red line on the right side of the scale. In this position the apparatus is ready for use.



FIGURE 2 General view of microelectrothermometer

To produce low temperatures, a mixture consisting of snow or crushed ice and salt is used (one part of salt and three parts of ice). The ice-salt mixture is placed in a 1000 or 500 ml jar covered with a metallic, painted lid (Fig. 2) with a hole in the center for the test tube containing the plasma sample (Fig. 4). Double walls of the test tube ensure a greater inertia in changes of plasma temperature. With the aid of a capillary pipet, one or two drops of plasma are discharged into the tube (Fig. 4). The test tube is placed in the cooling mixture and the working part of the microthermometer is immersed in plasma. The switch on the right side is set on the position "temperature".

With cooling of the plasma in the test tube, the scale pointer of the thermometer commences to move towards the left. The plasma becomes supercooled and the pointer, moving to the left, indicates the temperature of the supercooled plasma. An ice crystal is then taken from the wall of the jar with the cooling mixture and is placed by means of a thin rod into the test tube with plasma. The pointer commences to move rapidly towards the right and stops at the temperature which is the freezing point of the plasma. Determinations are usually carried out in triplicate, and the arithmetic mean

is calculated. To obtain pure plasma, the blood sample is centrifuged or left to separate in small test tubes.



FIGURE 3. Panel of microelectrothermometer

To determine the accuracy of determinations of the freezing point, (A) of sodium chloride solutions of known concentrations were measured.

It can be seen from Table I that the accuracy of the microthermometer used is quite high.

In Table II comparative data are presented on the freezing point of plasma of some fish. Data obtained by other authors with the aid of Beckmann thermometer are compared with our data obtained with the aid of the microelectrothermometer.

The scale graduation of the microthermometer should be checked periodically. For this purpose bidistilled water and 1% solution of chemically pure NaCl are used. The freezing point of bidistilled water is at  $0^{\circ}\text{C}$ , and that of 1% solution of sodium chloride at  $-0.630^{\circ}\text{C}$ . The freezing point of 1% solution of sodium chloride is given in the literature and is accounted for by electrolytic dissociation at the given temperature. When the readings show deviations from these figures, the apparatus should be readjusted or the determinations should be corrected for the factor found

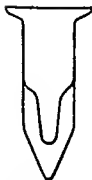


FIGURE 4 Test tube for sample (plasma)



The new method of determination of the freezing point of plasma with the aid of the microthermometer saves time and work, and yields almost accurate and objective results. Serial production of this apparatus will facilitate the rapid accumulation of data on osmotic regulation and salt metabolism in animals.

TABLE 1  
Freezing point of NaCl solutions

NaCl concentration, %	$\Delta$ , calculated	$\Delta$ determined with the aid of microelectrothermometer	Deviation from calculated
2.5	-0.157	-0.150	-0.007
5.0	-0.315	-0.306	-0.009
7.0	-0.441	-0.437	-0.004
10.0	-0.630	-0.618	-0.012
12.0	-0.756	-0.762	+0.006
15.0	-0.945	-0.920	+0.025

TABLE 2  
Comparative data on  $\Delta$  of blood of some fish

Fish	Blood serum	Author
Tench	0.49	Dekulzen
"	0.52	Case
"	0.54	Puschel
"	0.50	Our data
Pike	0.53	Dekulzen
"	0.51	Case
"	0.52	Our data
Perch	0.51	Dekulzen
"	0.45	Our data

## METHODS OF STIMULATION OF OVULATION IN FISH

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In fish, as in higher vertebrates, maturation of the sex organs is considerably stimulated by hypophyseal gonadotropic hormone. Under the influence of this hormone ovulation in fish is also stimulated. Administration of gonadotropic hormone brings about ovulation outside the spawning period, however, eggs capable of normal development are produced only when the fish gonads are in the IVth stage of maturation.

It was shown by N. L. Gerbil'skii that the gonadotropic hormone of the hypophysis is specific to fish families. Thus, to be sure of results one must use hormones obtained from the same or closely related fish species.

The activity of the gonadotropic hormone of fish hypophysis differs, depending on the season of the year. For stimulation of ovulation, hormone obtained in winter or during the spring spawning season should be used. To stimulate ovulation it is necessary to administer a suspension of fresh fish hypophyses or hypophyses specially treated for prolonged storage in an active state. This method necessitates the excision of fish hypophyses, which are placed in acetone [hypophysis: acetone - 1:10(20) (w/v)] kept for 12 hours in a glass vessel provided with a ground glass stopper. The acetone is then decanted, a new portion of acetone is added, and the mixture is kept for another 6-8 hours. The hypophyses are then taken out of the vessel and dried on a filter paper. Hypophyses prepared in this way may be stored in stoppered bottles or test tubes. In acetone-treated hypophyses the gonadotropic hormone may remain active for several years.

To induce ovulation, fresh or acetone-treated hypophyses are ground in a porcelain mortar with a small amount (0.5-1.0 ml) of saline (0.65% of NaCl). The suspension obtained is injected into the back muscles of the fish, approximately at the border between the anterior and middle thirds of the fish body. 1-2 hypophyses are usually injected into each fish. In fish kept in water with a temperature suitable for spawning, ovulation takes place 20-50 hours after administration of hypophyseal extract. Ovulation is determined through repeated examinations of the fish in question.

Similar injections of hypophyses in the same doses are used for obtaining nature spermatozoa from male fish.

Injection of peptone also induces ovulation in prespawning females. For this purpose 11-20% solution of peptone in distilled water is prepared. Dosage: 1 ml injection into abdominal cavity per female weighing 250-300 g.

Simultaneous intramuscular injection of hypophyseal suspensions, and intra-abdominal injection of peptone, considerably enhance the effectiveness of gonadotropic activity of hypophyseal suspensions.

Among the essential drawbacks of this method is the additional stimulation caused by the thread itself, which tends to distort the picture of motor activity of the fish, at least during the first days of the experiment. In addition, not all movements are recorded, but mainly circular movements, which cause the end of the thread to become entangled, so that the recorder will deviate away from the drum. A method free of these drawbacks was proposed by Jones (1955). Water fluctuations appearing during motion of the fish are conveyed to a light ebonite plate placed in the water (Fig. 2). Even a slight shift of the plate causes closure of the electric circuit. Each circuit closure represented one movement, and was registered on a kymograph or on a counter. The possibility of quantitative recording of movements of free-swimming fish enabled us to use this method for determination of the threshold of light perception (Woodhead, 1956). This method can thus be used for studies on motor activity in general and for studies on movements caused by some stimulants, i.e. it can be used for studies on receptor functions\*.

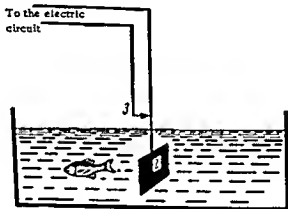


FIGURE 2 Apparatus for registration of motor activity in fish

1- aquarium, 2- ebonite plate, 3- electric switch

#### REACTIONS OF FISH TO ELECTRIC LIGHT

Reactions of fish in a field of a direct current has been studied in detail by Scheminsky (1936). He distinguished five different stages of reaction in fish to a gradual increase in the strength of direct current. During threshold current strength, a primary initiating reaction is noted. With an increase in the strength of the current, the fish commences to move in the direction of the anode - the stage of galvanotaxis. In the next stage, the stage of galvanonarcosis, the fish lies on its side with its head directed towards the anode. Active movements cease, the muscles are flaccid. Further increase in current strength leads to immobilization and contraction of all muscles. Finally, in the last stage, the fish dies. Of the reaction

\* For the description of other methods of registration of motor activity see below, pp. 274-276

mentioned above, the most interesting is the stage of galvanotaxis which may cause the directed movement of a large number of fish at one and the same time. It is true that fishing on a large scale with the aid of electric current is not yet practicable (Chernigin, 1956), since it calls for a large expenditure of electric energy. Further studies on the mechanism of galvanotaxis appearing under the influence of currents of various form and duration may eventually result in a practicable method of fishing with the aid of electric current.

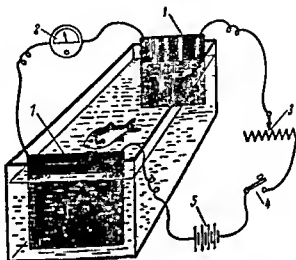


FIGURE 3. Apparatus for studies on the reaction of fish to electric current

1 - electrode, 2 - voltmeter, 3 - rheostat, 4 - key, 5 - accumulator

The method of study of galvanotaxis is a very simple one and its physiological and technical principles have not changed since the phenomenon of galvanotaxis was first discovered. An aquarium with experimental fish is provided with two plate electrodes (Fig. 3). The electrodes are most frequently arranged along the entire width of the aquarium at the opposite aquarium walls, so that the entire space of the aquarium is acted on by the electric current. Should the studies also include the behavior of fish outside the electric field, the width of the plate electrodes should be less than the width of the aquarium. Experiments on galvanotaxis are usually carried out on marine fish, because sea water has a greater electric conductivity than fresh water (electrodes should be prepared from corrosion-resistant material). To prevent electrode oxidation they are usually prepared from carbon or lead. The circuit should include a rheostat for changing current strength, as well as a voltmeter and an ammeter. To determine the strength of the current acting on the fish in question, the area of the electrode immersed in water and the distance between the electrodes must be known. Numerous authors have shown that electric sensitivity of fish to electric current is species specific and depends on the size of the

## EXPERIMENTAL METHODS OF STUDY OF FISH BEHAVIOR

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Thanks to technical achievements the underwater world hitherto hidden from the human eye became accessible to direct observations. The use of sounding devices, aqualungs, underwater television and other similar methods has enabled scientists to study the behavior of fish in their natural environment. However, for the detailed analysis of physiological mechanisms of fish behavior it is often necessary to study their behavior under experimental conditions which allow for an accurate dosage and control of factors affecting fish behavior and registration of the reactions of fish to these factors. Under natural conditions this is impossible. This chapter is devoted to the description of such experimental methods.

Under the term "behavior" physiologists include both the unconditioned, inborn reactions of animals to external and internal stimuli, and the conditioned reactions acquired during life. Both reactions are closely interwoven. There are no pure inborn and pure acquired reactions. For certain purposes however, they can be studied separately. Hence the methods for study of animal behavior are divided into two: a) methods of study of unconditioned reflexes, i.e. the inborn component of fish behavior and b) methods of study of conditioned reflexes, i.e. the acquired component of behavior. The first part includes the description of 1) recording of motor activity; 2) methods of study of the reaction of the fish to electric current, 3) methods of study of the reaction of fish to light, 4) methods of study of the reflex of following; and 5) methods of preference. The second part includes a description of techniques for studies of conditioned defense and alimentary reflexes, and also the description of the labyrinth method for studies on fish behavior.

The dynamics of nervous processes, which are the basis of any behavior act, are usually judged by changes in the activity of muscles or glands. The activity of the nervous system of fish may at present be studied directly by recording biocurrents from various segments of the nervous system by means of electrodes inserted for prolonged periods. This method is not yet widespread, the reason for it is the complexity of the method, and what is more important, for such studies the fish must be kept under conditions which differ greatly from their natural habitat. The electrophysiological method may prove of value as an auxiliary method in studies on higher nervous activity of fish in combination with the method of conditioned reflexes.

### MOTOR ACTIVITY

A very common method of recording fish behavior involves the measurement of its motor activity, including alimentary, sexual, defense and other

unconditioned and conditioned reflexes. Data on the distribution of these activities during one day, and on the different factors affecting the activities of fish are of great importance for the correct organization of fishing. Motor activity can be studied under natural conditions, by the depth from which different fish can be caught during various periods of the day, from their feeding activity, etc. In spite of this, the multitude of factors which remain unaccounted for render the results obtained under natural conditions unreliable. Under experimental conditions, motor activity also changes but its dependence on certain factors can be determined fairly accurately. For a complete understanding of motor activity of fish it is thus essential to compare the data obtained under natural and experimental (artificial) conditions.

Changes in motor activity of fish may be studied by means of the simple method of Szymanski (1914). The fish to be studied is placed in a small vessel filled with water (Fig. 1). A thread is fixed on the dorsal or caudal fin. One end of the thread is connected with a light lever, fixed on an axis, the height of the lever in relation to the fish can be adjusted by moving it along the tripod. The lever is balanced so that any movement of the fish is recorded on a blackened kymograph drum, by means of a pointer attached to the long arm of the lever. The rate of revolutions of the drum may be adjusted depending on the duration of the experiment. For recording the activity of fish during one day, barograph drums should be used (or a drum of some other similar apparatus). Quantitative evaluation in this method is difficult, because the movements may be so frequent that all marks merge into one continuous line, so that the number of movements cannot be counted and the magnitude of one movement cannot be compared with that of another movement. In spite of the fact that this method is a primitive one, it allows one to establish the daily rhythm of motor activity in fish. Shtefan (1957), by using this method, showed that the motor activity of sticklebacks in shoals is less pronounced than that of isolated fish.

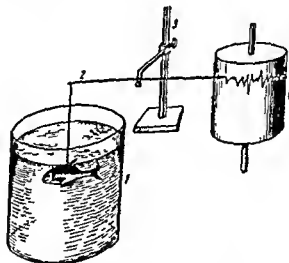


FIGURE 1 Apparatus for studies on motor activity in fish  
1 - aquarium; 2 - lever; 3 - tripod; 4 - kymograph

fish. Thus, in experiments on galvanotaxis the length of the experimental fish must be measured.

In studying the galvanotaxis of fish one must remember that the reaction of the fish depends on the distance between its body and each of the electrodes, as well as on the location of its body axis in relation to the plane of the electrodes.

Present studies on galvanotaxis aim at a more effective use of electric current. It has thus been found that direct current applied in impulses has a greater effect on fish than continuous current. Studies on the effect of currents of varying frequency and form are of great practical importance; the solution of this problem will enable us to use electric current in large-scale fishing. Reactions of fish to electric current may be a subject not only for applied studies but also for theoretical investigations, since they serve as a subtle criterion of the functional state of the nervous system of the fish. To determine the sensitivity of fish to electric current, attention should be directed to the first stage of its external manifestation, the primary reaction. Using this method the effect of various chemical and physical factors on the nervous system may be studied. It has thus been shown that ethanol and magnesium chloride increase the sensitivity of fish to constant electric current, whereas caffeine decreases this sensitivity (Puchkov, 1954). It has been shown that light increases the sensitivity of fish to electric current while a magnetic field lowers it.

#### REACTION OF FISH TO LIGHT

Among the natural physical stimuli, light is one of the most important factors predetermining the behavior of fish. Even such a rough indicator as movement towards or from a light source, i.e. positive or negative phototaxis, shows that almost all fish react to light. The use of such methods as recording of motor activity, measurements of sensitivity to electric current, change of conditioned reflexes, etc. provide comprehensive data on the effect of light on fish behavior. From the practical point of view, the method for determination of the direction and intensity of phototaxis in fish is of great importance. Following is a description of the method reported by T.I. Privol'nev (1956).

Fish of the type *Leucaspius delineatus*, up to 20-25 specimens, are placed in an aquarium one half of which is darkened by plywood while the other half is exposed to light (Fig. 4). These sides (the darkened and illuminated one) are joined by an opening in the partition. One hour after the fish are placed in the aquarium, the number of fish present in the light part of the aquarium are counted. Subtracting this number from the total number of fish, the number of fish present in the darkened part of the aquarium can be calculated. For example, experiments with European cisco. In 68% of cases this fish were present in the part exposed to light, and 32% in the dark part. The intensity of reaction of this fish to light will be expressed as  $68 - 32 = 36\%$ . Similar experiments with eels and lampreys showed that these fish, in contrast to others, always avoid light. In spring and autumn, "verkhovka" (*Leucaspius delineatus*) follows in the direction of light, and in winter avoids it. Positive phototaxis is strongly pronounced in fry of numerous fish species.

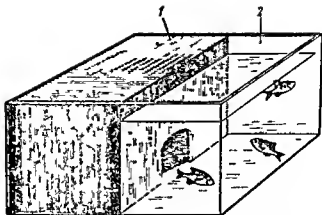


FIGURE 4 Aquarium adapted for studies on the reaction of fish to light  
1 darkened part of the aquarium 2 part exposed to light

Species, age and seasonal differences play a part in the reaction of the fish to light and again stress the complexity of the effect of this factor on the behavior of fish. No unified theory is at hand which could explain the biological significance and physiological mechanisms of phototaxis. Light is frequently considered as a positive conditioned alimentary stimulus, but this assumption does not explain many instances of phototaxis, including the positive reaction to light in fry immediately after their hatching from eggs. The mechanism of the effect of light should be investigated for practical purposes, since the empirical application of this factor has been instrumental in increasing the catch of certain fish (Borisov, 1952).

#### METHOD OF PREFERENCE\*

In studies on galvanotaxis and phototaxis only the reaction of the organism to one stimulus is recorded, i.e. motion towards or from the stimulus under natural conditions. The fish are, however, exposed to several factors (visual, thermal and chemical). For studies on the effect of these factors the method of "preference" is used. To illustrate this method, let us describe the apparatus proposed by T. I. Privol'nev (1956), who studied the reactions of fish to various light waves of the visible spectrum.

Fish (20-25) are placed in an aquarium divided into 5 compartments by 7 detachable partitions (Fig. 5). Each compartment is covered with glass of a different color. Thus in experiments with European cisco the compartments were covered with the following: 1) colorless glass, 2) red glass, 3) green glass, 4) blue glass, 5) opaque glass. After the fish have been kept for 10-15 min under an illumination of varying wavelength, the partitions were lowered and the number of fish present in each compartment were counted. The experiments showed that European cisco avoids compartments covered with red glass, preferring compartments covered with colorless or green glass. The behavior of fish of other species differed from that of the European cisco.

The method described above is a rough one both in light gradation and in the determination of fish distribution; nevertheless this example indicates



the principle of the "preference" method. The accuracy of this method can be increased depending on the aim of the studies and on the technical devices used. Thus, in determining the "preference" of extent of illumination, one end of a long aquarium may be brightly lit, and the intensity of light should diminish gradually to complete darkness at the second end of aquarium. In addition, the aquarium can be divided not only into five, but into more numerous compartments.

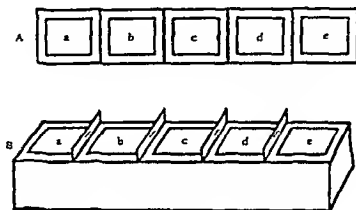


FIGURE 5 Apparatus for studies on the reaction of "preference"

A - view from above B - general view, a b, c d e - compartments f - partition

The method of "preference" may be used successfully in studies on the function of chemo- and thermoreceptors. In this case a gradient of the factor studied may be created, i.e. temperature gradient, or a gradient of concentrations of substances dissolved in water. The distribution of fish is determined in the same way, irrespective of the stimulant used. It is important to form a gradient of the stimulus used, and to exclude the effect of other similar stimuli. Thus, for example, in experiments on the effect of light on fish behavior it is important that light should not warm up the water in the aquarium

#### REFLEX OF FOLLOWING

Reflex of following is the following of one fish by another. This reflex apparently comprises an optokinetic reaction, i.e. motion of an animal after some moving object. This reflex is often more complex, since experiments have shown that movement of a dummy fish of the same species causes a stronger reflex than movement of a dummy of a different fish species. This seems to indicate that the reflex of following is an elementary imitative reflex, playing a major role in shoal behavior of fish. Studies of this reflex may lead to determination of its biological importance, its interrelationships with other shoal reflexes, the role of the visual analyzer in its realization, etc.

The method of study of the reflex of following was elaborated by L. B. Kozarovitskii in the laboratory of L. G. Voronin. The main part of the

apparatus for studies on the reflex of following is a rectangular frame on the edges of which two pivots with pulleys are fixed (Fig. 6). Belt-drives made from a fishing line pass through the pulleys. To the fishing line tin dummy fish of the studied species are fixed at desired intervals. The number of pulleys may vary, depending on the number of dummy fish required (density of dummy fish shoal). The entire system is set in motion by an electric motor located above the water near one of the pivots. Above this leading pivot there is another pulley (above water) connected with the electric motor reduction gear. The movement of the electric motor is conveyed to the leading pivot, which with the aid of pulleys pulls all fishing lines, and the shoal of dummy fish commences to move around the frame. In order that the fish should not see the dummies moving along the opposite side of the frame (dummy fish moving in the opposite direction) the front of the frame is separated from the back by an oil-cloth.

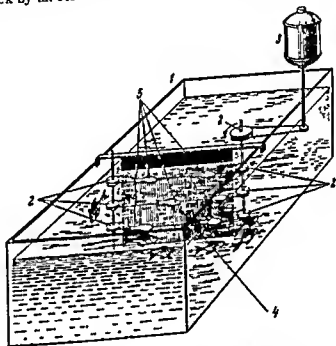


FIGURE 6 Apparatus for studies on the reflex of following in fish  
1 - aquarium; 2 - pulley; 3 - electric motor; 4 - partition; 5 - dummy fish

In an aquarium with a shoal of fish of one and the same species the frame is located so as to divide the aquarium in two equal parts, leaving a small narrow space between the frame and the side walls of the aquarium. For the quantitative evaluation of the reflex of following, the number of fish passing along both sides of the frame in a unit of time is counted. Care should be taken that both halves of the aquarium will not differ from each other in the intensity of illumination and other properties which may affect the distribution of fish. In addition, it should be remembered that fish aside of the frame will represent a more adequate stimulus, and thus inhibit the reflex

of following after the moving dummy fish. Studies on the reflex of following in crucians and "verkhovka" showed that this reaction was noted both in shoal and non-shoal fish, being more pronounced in shoal fish.

#### METHODS FOR STUDIES OF CONDITIONED REFLEXES IN FISH

The first studies on fish behavior carried out by means of the method of conditioned reflexes were carried out by Pavlov's pupil, Yu. P. Frolov (1923). During the following 25 years the function of the sensory organs in fish, and especially the function of higher nervous activity of the fish, had been studied successfully by means of Pavlov's method. This method opened up wide possibilities of study, as indicated by recent progress in studies on the analysis of lateral line in fish (Malyukina, 1955), and on reflexes and their relationship to stimuli of all types (Chumak, 1957), on complex chain reflexes (Prazdnikova, 1955; Taglev, 1957; Vedyayev, 1956), and on imitative reflexes (Bogomolova, Saakyan and Kozarovitskii, 1958).

The method of conditioned reflexes has many things in common with the training successfully used by zoopsychologists in their studies on fish behavior from the beginning of this century. The common thing is the objectivity of the principle. The investigator records the interaction between the organism and its external environment in the animal's attempt to overcome the problem posed by the investigator. The difference lies in the fact that zoopsychologists do not register the magnitude of the stimulus, its quality, force and the peculiarity of response of each organism; they do not compare the properties of stimulation and response, thus being unable to solve the problem of nervous mechanisms of behavior.

The main rule of the method of conditioned reflexes is the simultaneous action (combination) of an indifferent agent and that of a stimulus causing some motor reaction in fish. The prerequisite condition is a careful checking of the indifference of the factor converted into a conditioned stimulus. Until the development of conditioned reflexes this agent should not, per se, cause the movement brought about by the unconditioned stimulus. The interval between application of stimuli may vary: most often one stimulus follows another in the course of 1 - 2 min. Depending on the nature of the unconditioned stimulus ("reinforcement") the methods making use of conditioned reflexes may be divided into alimentary and defense ones. The number of combinations in one experiment is determined by the nature of the reinforcement, the size of the fish, and other factors, but in most cases 7 - 15 combinations are sufficient to produce the desirable effect.

Objective recording of experimental conditions is of great importance in studies on conditioned reflexes carried out either by the defense or alimentary method. Of course, the main document is the protocol, where the number of the experiment, its duration, the ordinal number of combinations, the nature of the conditioned stimulus and the duration of its application, the delay in the appearance of conditioned reaction, and the nature of reinforcement are all recorded. The protocol usually includes kymograms of the most important elements of the experiment. In the upper line of a kymogram (Fig. 7) the conditioned reaction is registered, on the following line below, the duration of application of the conditioned stimulus is plotted. Still lower, the time of application of the unconditioned stimulus, and finally on the lowest line the time, are recorded.

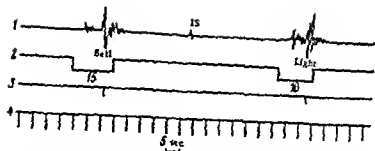


FIGURE 7 Kymogram of an experiment on the development of a conditioned electric defense reflex to light and sound

1 - recording of motor activity of fish 2 - conditioned stimulus 3 - unconditioned stimulus 4 - time 15 - interval motor activity

#### ELECTRIC DEFENSE METHOD

The earliest (Frolov, 1941) and most widely used method for studies on conditioned reflexes in fish is the electric defense method (Fig. 8). Movements of the fish placed in a small aquarium are registered on a kymograph with the aid of two Marey capsules. The thread is fixed at one end to the lever of the first capsule, and at the second end to the dorsal fin of the fish. The movements of the fish cause oscillations of the membrane of the first capsule. These oscillations are conveyed to the second capsule by means of a rubber tube. The second capsule is connected with a lever which in its turn is provided with a pointer for recording fish movements on a kymograph drum. The current of accumulators serves as an unconditioned stimulus, the former is fed to electrodes through an induction coil. One electrode is made of a metal plate placed on the floor of aquarium, and the second electrode is a wire fixed to the dorsal fin of the fish. By moving the induction coil, one may select a stimulus of such force that it will only slightly exceed the threshold current. The light of an electric lamp is filtered through various color filters, and a sound from a conventional small electric bell suspended above the aquarium may serve as conditioned stimuli. Sound can also be produced with the aid of a telephone apparatus membrane sealed in a waterproof case and immersed in water. In studies on the action of chemical, thermal, infrasound, magnetic and other stimuli, the method becomes complicated only by the necessity of constructing additional devices which ensure the creation of conditioned stimuli.

The principle of electric defense methods proposed by Yu. P. Frolov has changed little since its first description, but the methods of unconditioned stimulation were modified along two lines. Bull (1928) and later numerous other investigators have placed both electrodes (in the form of plates) on the aquarium walls. S. N. Kirillov (1936), on the other hand, placed both electrodes in direct contact with the body of the fish (local stimulation). Owing to the simplicity of the method and to the fact that the unconditioned stimulus may be applied in desired doses, the electric defense method has

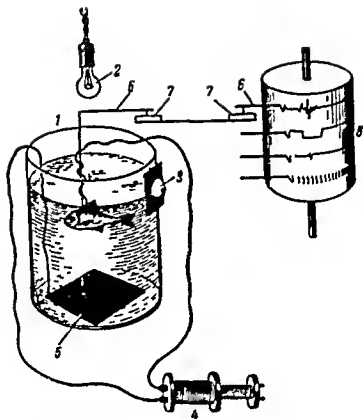


FIGURE 8 Diagram of apparatus for elaboration of conditioned electric defense reflexes in fish

1- aquarium, 2- light stimulus, 3- sound stimulus 4- induction coil 5- electrode 6- lever, 7- Marey capsule, 8- kymograph

been widely used in comparative studies on the mechanisms of higher nervous activity and on the effect on it of various factors. However, the marked increase in excitation of the nervous system caused by an electric stimulus restricts the use of this method. For this reason, mechanical or chemical unconditioned stimuli are preferred at present (p. 210 - 213).

#### OTHER DEFENSE METHODS

By combining any indifferent agent with a mechanical stimulus caused by a strong stream of air or a slight tap with a glass rod, one can bring about a development of a conditioned defense reflex. Thus, G.V. Popov (1953) combined the effect of light and air stream under the influence of which fish fry descended to the bottom of the aquarium and attempted to obtain a conditioned defense reflex in fish fry. After several applications of the combined action of air and light, the fish fry began to descend to the bottom of aquarium under the influence of light.

On the basis of mechanical stimulation it is possible to develop conditioned reactions which consist in the fish's swimming over from one half of the aquarium to the other (Fig. 9).\*

\* See the following chapter by N. V. Priznalkova

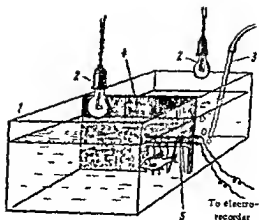


FIGURE 9 Apparatus for the development of conditioned defense reflexes in fish to mechanical unconditioned reinforcement

1 - aquarium, 2 - light stimulus, 3 - unconditioned stimulus (stream of air), 4 - partition, 5 - movement recorder

Artificially elevated pressure in the swimbladder may be used as an unconditioned stimulus which caused a motor reaction in the fish (Sokolov, 1953). The same purpose is served by the addition of  $\text{CO}_2$  to the aquarium, which results in a changed rate of respiratory movements of the fish (Chernova, 1953, see also the following chapter by N. V. Prazdnikova).

#### ALIMENTARY METHODS

Alimentary reinforcement has a certain advantage over the electric one by being more natural and because it has no deleterious sequelae. Bull (1928) used the alimentary method for elaborating conditioned reflexes, but his studies lacked an objective registration of conditioned reactions. The investigator noted visually how the fish swam to the place of feeding or to its exit from the shelter in response to the action of a conditioned stimulus.

V. A. Pegel' (1950) developed conditioned alimentary reflexes in fish by using the same principle as Yu. P. Frolov in his studies on defense reflexes. Fish movements were registered with the aid of a Marty's capsule which was connected to the dorsal fin by a thread. During application of the conditioned stimulus the fish obtained food in a certain place. To reach this place the thread fixed on the fish had to be stretched to its limit. After several combinations of electric light with feeding, the fish began to move to the place of feeding immediately after switching the light on. Although this method allowed for the objective recording of conditioned reactions of the fish, the thread itself caused additional stimulations which increased the number of movements, thus making the detection of conditioned reactions difficult.

According to the method of Herter (1953), the fish also responded to conditioned stimuli by swimming over to the feeding place. Herter gave the fish two objects to choose from: 1) food - mostly cheese and 2) dummy food-wax which in form, color and smell resembled cheese. Both objects

were offered simultaneously in the form of small pieces fixed on two widely set prongs of one and the same fork. This was accompanied by the application of various conditioned stimuli (i.e. cheese was placed against the background of a green triangle, and the wax against a red background). After several combinations the fish always swam to the green triangles, irrespective of the direction from which they came. The drawback of this method, apart of objective registration, lies in the simultaneous development of positive conditioned reflexes and differentiation. However, this method gives an adequate objective characterization of the ability of fish to differentiate color stimuli.

A method making use of conditions more closely related to natural ones has been developed by Prazdnikova (1953) in the laboratory of L. G. Voronin. It was noted that fish frequently take inedible particles (plant remnants, pebbles, sand, etc.) into their mouths and then eject them. When a bead was placed in the aquarium, the fish caught it and immediately ejected it. This reaction was repeated several times and then the fish ceased to catch the object. This reaction was clearly of an exploratory-alimentary nature. If this reaction is reinforced by feeding it soon becomes a conditioned reflex (Prazdnikova, 1953)\*.

The alimentary method has the following advantages over the electric defense method 1) the motor reaction on the basis of which the conditioned reflex is elaborated is natural to the fish, 2) the fish remains all the time under unchanging conditions, 3) on regulating the magnitude of alimentary reinforcement to satiation of the fish, it is possible to use a large number of combinations of various stimuli.

#### LABYRINTH METHOD

The labyrinth method has been used by zoopsychologists for elucidation of the effect of various factors on the "training capacity" of fish. The principle of this method lies in the fact that different obstacles are placed between the fish and the food, and that the time required to reach the food in the first and following experiment is recorded. This experiment is repeated several times, and the reduction in the time required to reach the food is noted. At the beginning of the experiment the fish is placed in the first compartment of the aquarium and food in the third compartment (Fig. 10). The time needed to reach the food in the third compartment is recorded with a stopwatch. After several experiments the fish begins to swim with an increasing speed from the first to the third compartment. Graphs of "training capacity" can thus be plotted. In these graphs the time required to reach the food is plotted along the ordinate, and the ordinal number of experiments is recorded along the abscissa. Similar graphs are plotted for each fish and compared.

The labyrinth method has not been widely used, since its results are difficult to interpret, while the general data on "training capacity" i.e., on the speed and kinetics of formation of conditioned reflexes can easily be studied by means of the conditioned reflex method. However, the labyrinth method is an objective scientific method which may prove useful for studies on fish behavior.

\* See the following paper written by Prazdnikova

Our short review deals only with the commonest methods used. It remains not only to improve the old methods, but to introduce new methods for studies on the behavior of fish under natural conditions.

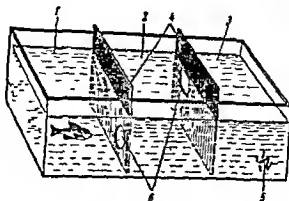


FIGURE 10 Diagram of labyrinth method for studies on fish behavior

1 2, 3 - compartment, 4 partition 5 food 6 - opening

Laboratory experiments form the basis for studies on fish behavior, and will provide methods of investigation of receptor capacity in fish, and the systematization of their unconditioned and conditioned reflexes, as well as allow to determine the relationship between inborn and acquired components of behavior. Of course these methods do not embrace the whole complexity of the problem. To this end, experiments under both laboratory and natural conditions should be carried out. Experiments under natural conditions should be designed on the basis of knowledge acquired in the laboratory, and from careful observations of fish behavior. Experiments under natural conditions should foresee any possible changes and hence aid in the control of behavior of shoal and isolated fish.

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## METHODS OF STUDY OF CONDITIONED REFLEXES IN FISH

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Studies on fish behavior by means of methods of conditioned reflexes reveal the rules governing the activity of the CNS in fish in general, and the mode of action of various analysors in particular. For this purpose, various methods may be used, but with maximum attention paid to the biological aspects of the organism studied.

The first studies on conditioned reflexes in fish are those of Yu P Frolov (1925, 1926, 1929, 1939, 1941), who used the methods formulated by him in studies on conditioned motor defense reflexes with unconditioned reinforcement by electric current. Somewhat later, Bull (1929, 1936, 1936a) used the defense method (similar to that developed by Frolov) in his studies. Since then a wealth of data on fish behavior studied by means of conditioned motor alimentary and defense reflexes have accumulated (Kirillov, 1936, Pegel', 1951, Karamyan, 1953, Baru, 1951, Prazdnikova, 1953 and others). A description will now be given of several methods applied in studies on higher nervous activity of fish by means of conditioned reflexes.

### METHOD OF CONDITIONED ALIMENTARY MOTOR REFLEXES

One of the most adequate methods is that of conditioned alimentary motor reflexes. Since motor activity and the ways by which fish obtain food are extremely divergent, various motor reactions of fish may serve as criteria of conditioned reflexes. Methods based on alimentary reflexes must fulfil the following conditions:

- 1) the conditioned motor reflex must be clearly pronounced,
- 2) if motor activity consists of swimming over to the feeding dish, the fish should not be allowed to remain near the feeding rack at other times,
- 3) fish movements should be as unhampered as possible.

In the literature two methods are described which fulfil these requirements. One is the method suggested by Bull (1951) for studies on the degree of differentiation by marine fish of various hydrographic environmental factors, such as temperature, salinity and pH of the water. The other method has been developed in the Laboratory of Comparative Physiology at

the Institute of Physiology im I P Pavlov of the Academy of Sciences of the U S S R (Prazdnikova, 1953) for studies on nervous processes in carps (omnivorous)

On the grounds of various tests, Bull proposed to use long, narrow aquaria with sloping bottoms, for such experiments. Owing to the slope (1:30) there is a depth gradient, no water or a shallow layer at one end of the aquarium where the feeding rack is placed, and a deeper layer of water sufficient to hold the fish at the other end of the aquarium. Under conditions of starvation, when the food is gradually shifted towards the shallow end of the aquarium, the fish gradually learns to swim the entire length of the aquarium to the site where it receives the food and immediately returns to the deep end of the aquarium. Changes in water temperature, salinity and pH of water were used as conditioned signals by Bull. Food served as unconditioned reinforcement. A scheme of a setup for studies on the effect of various temperatures is presented in Figure 1.

The observer sits at point 1, he is separated from two large aquariums (upper part of the figure) and 6 smaller aquariums by opaque screens (2 and 3). Sea water from the laboratory reserves is fed into the system through tube (4), it flows along each aquarium and overflows through the edge of the deep end. The inlet of tube (5) through which food is introduced into the feeding compartment of each aquarium is shown by the dotted line. Periscopes (6) are mounted in the screens. These allow one to inspect the entire length of all aquariums clearly. Small heating coils (7) allow for a slight increase in the temperature of water flowing through them. This is accomplished by the pushing button (8). Water flow may be followed with the aid of thermocouples connected with a sensitive galvanometer (9). The investigator is capable of carrying out all necessary procedures noiselessly, without having to move.

Bull described the experiment thus: 'The investigator enters the building and closes both doors. He inspects the aquariums to ensure a faultless flow of water and to see whether everything is in order. The investigator then takes a seat on chair (1) and records the behavior and location of the fish for at least 15 minutes. The temperatures on the border of cold water and the galvanometer reading of each thermocouple must be registered. The heating device is then switched off and galvanometer readings are recorded. At each association a piece of food is placed in the feeding compartment. During several first experiments the fish does not respond to the first stimulus—food—present in the feeding compartment, there is no reaction until the fish perceives it (visual perception, smell perception, etc., depending on the feeding habits of the fish studied). Cod, for example, do not respond for several minutes (i.e., until they have perceived food by smell, taste or sight, the latter is less probable) because of the distance at which the food is placed. After the fish has perceived the food it immediately commences to move towards it, against the current, and continues to swim to the place (half-way to food) where the water layer is too shallow for the comfort of the cod. At the early stages of the experiment the fish immediately returns to its starting point, but soon it commences to swim against the current, again returns and so on. Fish movements gradually become more and more excited (with each approach to the shallow water and to the food), so that it gradually penetrates shallower waters. Finally, the fish makes a rapid splashing movement, enters the feeding compartment and attempts to obtain the food placed there, acting, apparently, in a state of extreme

excitation At first, the fish usually does not succeed in snatching the food, and splashing in the shallow water, it finally returns without the food it had attempted to obtain In the course of several days the fish accomplishes these movements not more than once a day, although the food remains in the chamber all this time and the fish is allowed to snatch it. If the food remains uneaten at the end of the day it is taken away and replaced. Repeating this association day after day the fish becomes more and more persistent in its attempts to obtain the food, and becomes less excited by splashing in the shallow water Finally, it learns to snatch the food without hesitation, remaining nevertheless highly excited to the very end of the experiments, and returns excitedly and rapidly to the deep end of the aquarium. Finally, the time comes when the fish displays signs of "anticipation". In eels which maintain their position by slight undulating waving of the pectoral fins, the first sign of a conditioned reaction is manifested in the enhancement of this fin waving. The fish then commences to swim against the current for varying distances, and finally displays the full reaction (as described above) and snatches the food It must be stressed that now the fish displays all this activity before placing food in the chamber. In the last stages of the experiment the fish swims into the feeding chamber and waits outside the shallow water until the food is introduced, it then immediately snatches the food and with a splash returns to the deep end of the aquarium.

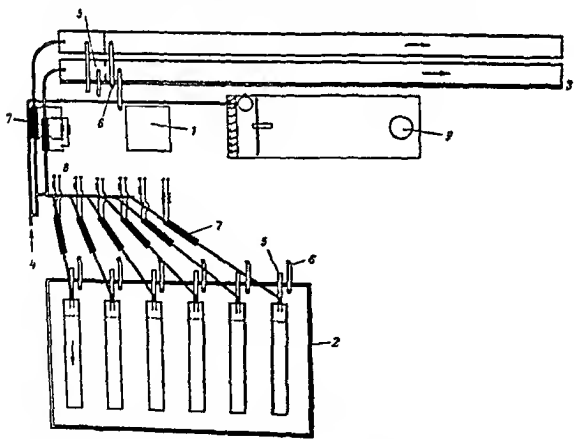


FIGURE 1 Apparatus used by Bull (1951) for developing conditioned motor reactions in response to a slight rise in temperature

During the entire procedure the fish is in a state of strong excitation, its movements are rapid, active and strong sometimes they are accompanied by masticating movements of the jaws. In sluggish fish such as *Blennius*, *Onos*, *Liparis*, etc., which under experimental conditions remain motionless for hours or even days on end, this reaction is even more striking

In the first experiments, conditioned reflexes were developed only in response to considerable changes in the factor studied. At first, the temperature was raised from  $0.4^{\circ}\text{C}$  to  $3.0^{\circ}\text{C}$ . After the conditioned reflex had developed the amplitude of the stimulus gradually decreased to the threshold perceived by the fish. It was shown that the fish *Gadus merlangus* and *Pleuronectes flesus* are capable of differentiating small changes in temperature, of the order of  $0.03 - 0.05^{\circ}\text{C}$ .

In his studies on the ability of fish to differentiate water according to the salt concentration, Bull proceeded from the hydraulic laws of water motion suggested for the creation of a uniform water flow, to use similar aquariums with sloping bottoms and with a L-shaped knee where the fish lives permanently. The fish in this aquarium swim with the water current. The current of water from the lower (deep) end to the upper end ensures a straight front of water flow along the entire aquarium and a uniform change in the water salinity. Apparatus used for studies on the effect of various salt concentrations on free swimming fish (cods, haddocks and others) is shown in Fig. 2. An approximate  $4\text{‰}$  change served as primary stimulus (from an initial salinity of  $34\text{‰}$  to  $30\text{‰}$ ). The gradual decrease in this difference allowed for observation of the conditional reflex when the salinity changes from  $0.5\text{‰}$  to  $0.2\text{‰}$ .

A similar aquarium was used for studies on the ability of fish to differentiate the pH of water. Change in pH was accomplished by adding to ordinary sea water at a pH of 7.95-8.22, a given amount of sea water, saturated with  $\text{CO}_2$  - pH 5.0-5.2. The change in pH of the water is from 7.95-8.22 to a pH of about 7.0 (addition of 500 ml of water saturated with  $\text{CO}_2$  to 10,000 ml of water in the aquarium and stirring) served as primary signal for the development of a conditioned reflex. Gradually, the fish learned to distinguish changes of the order of 0.05-0.07 pH units.

We shall now describe the second alimentary method.

The fish should live constantly in the aquarium. The feeding box is placed in one of the corners of the aquarium. The food is rinsed in water. A small bead fixed on a thread is placed in the water. One end of the thread is fixed to the feeding box. A conditioned reflex in the fish is manifest by a movement aimed at snatching the bead in response to a conditioned stimulus. The reflex is developed in the following way. The bead is suspended in water. The fish (carp family, omnivorous) in search of food takes all small objects in its mouth, including the bead (Fig. 3). This movement is immediately reinforced by placing food into the feeding box. After 15-20 experiments the fish will have developed a conditioned motor reflex. Having snatched the bead, it throws it away and immediately swims over to the feeding box. A stimulus is then used which should subsequently serve as a conditioned signal. Only movements coinciding with application of the stimulus are reinforced. Of course, this experiment should be carried out on hungry fish. The stimulus should be applied for not more than 15-30 seconds. In the course of the first or second experiment, the conditioned link-conditioned stimulus-jerk-food is established, the conditioned stimuli may be applied

30-50 times per single experiment, with 2-minute intervals. As a rule, the duration of application of the conditioned stimuli should be restricted to 15 seconds. The duration may be doubled (to 30 seconds) when the local conditioned reaction is delayed, but the general motor reaction of the fish is clear-cut (the fish swim over to the bead and performs a jerking movement).

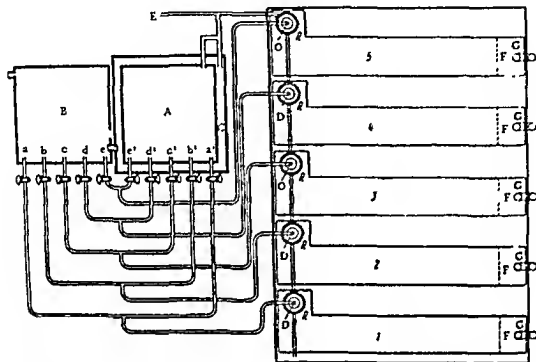


FIGURE 2. Apparatus for differentiation of salinity (Scale of the left-hand part is 1.6 times larger than scale of the right-hand part). (After Bull, 1951)

A—reservoir of water used for stimulation, B—reservoir with ordinary sea water, C—reservoir around reservoir A, a-d and a'-d'-tubes through which water passes into the sloping (1:30) aquariums (1-5), D—devices for water distribution ensuring a constant water level in the aquarium, E—faucet for sea water.

Reinforcement is applied not earlier than on the fifth second of duration of the stimulus. Food (mostly a butterfly, or a piece of earthworm) is given in such amounts so as not to exceed 50% of the daily portion (needed for full satiation) of the fish. After such an experiment the fish is given no further food.

The following conditioned signals may be used:

1) light signals—electric bulb of 75,100 or 250 watt suspended above the aquarium (Fig. 4, 2). By using light filters, light of various colors may be obtained. In experiments with fish of the carp family, yellow, green or blue lights are preferred. Red light is not recommended. Flickering light is a good stimulus. This can be obtained by placing a four-blade rotator fixed on the axis of a Warren motor which is then placed in front of a bulb of a 10-15 watt bulb. This device is suspended on the front wall of the aquarium;

2) sound stimuli—sound of an electric bell suspended over the aquarium, underwater oscillation of a tuning fork, buzzer, etc. It is important that

the source of sound should be placed in water or in the aquarium but not above the water surface. In experiments with the above conditioned stimulus the aquarium should be isolated from floor oscillations by placing it on holders.

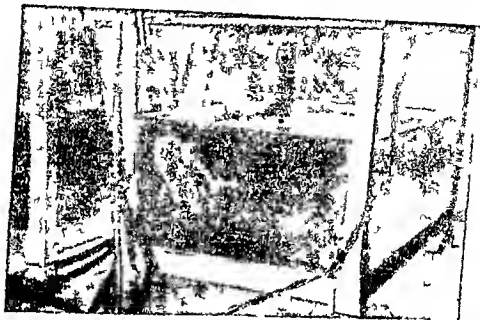


FIGURE 3 General view of an aquarium for studies on conditioned alimentary motor reflexes (Prasolova 1953)

3) bubbling produced by blowing air into water is a very good conditioned stimulus

In experiments with conditioned alimentary motor reflexes the following data should be recorded

1) jerk—recorded by means of closure of electric contacts connected with the bead

2) the beginning and end of application of the conditioned stimulus. For this purpose the storage battery circuit of the electromagnetic recorder is connected parallel to the main circuit of the stimuli by means of an I type key

3) time (every 5 seconds) is recorded by means of an electromagnetic recorder. The kymogram of such an experiment is shown in Figure 5

The following pointers will be useful for investigators interested in methods of conditioned alimentary reflexes

At the beginning of his study the investigator will find that fish of one and the same species, age and sex display individual fluctuations in behavior. Some fish are very active and begin to snatch the bead immediately. In that case the conditioned stimulus should best be employed already in the first experiment without preliminary elaboration of the motor reflex—snatching of the bead. In such fish a conditioned reflex to the stimulus is also rapidly developed and during the intervals between application of stimulus the jerks are less numerous than in case the motor reflex was



first developed. Sometimes fish are encountered with a sharply pronounced passive-defense reaction. Such fish display this kind of reaction towards the bead, and in the first days of the experiment towards food, dropped into the aquarium. With such fish, preliminary development of a motor reflex toward the bead is indispensable. Usually, first application of the conditioned stimulus causes inhibition of jerking which gradually passes, and the fish then always develop a conditioned reflex.

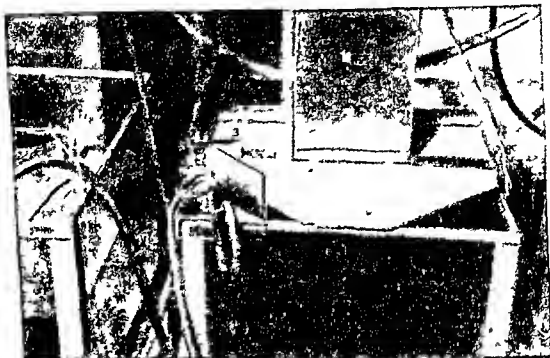


FIGURE 4 Upper part of aquarium with feeding rack (1) light source (2) and switches (3) for recording motor reflexes

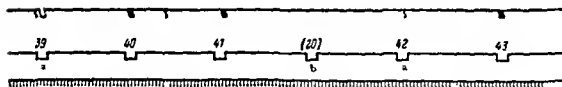


FIGURE 5 Kymogram of experiment

From the top: jerk mark stimulus mark time mark a - white light b - light green light

It is best to begin experiments in summer, because of the high alimentary excitability of fish in the summer. In a winter which follows a summer abounding in food the alimentary excitability of fish is lowered, and conditioned reflexes elaborated in winter may prove unstable and inconclusive.

In experiments with two conditioned signals—a positive and inhibitory one—the rhythmic stereotype with alternating stimuli (+-, +- etc.) and equal intervals between stimuli is recommended.

In experiments with predatory fish (for example, perches) the motor reaction described above is impractical. Being predatory fish, perches may lie motionless in wait for long periods near the feeding box and catch the food with a swift dash. Attempts to develop alimentary motor reflex—catching the bead—were unsuccessful. Only a general motor reaction could be developed—swimming over to the feeding box. So that the fish would not remain near the feeding box all the time, two feeding boxes should be constructed with local conditioned signals indicating the box in which food is placed. In such a case, swimming over to the feeding rack in response to a signal will mark the conditioned reaction. The following recording device is recommended (Fig. 6). At the entrance to the feeding box, metallic rods are suspended from a steel plate, another piece with holes for these rods is placed in a horizontal position about 10 cm below the first plate. Just above water surface the metallic rods are connected with glass rods to form a type of mobile curtain. Swimming towards the feeding box, the fish draws the rods apart, thus closing the contact of the electromagnetic recorder which records this movement on a kymograph.

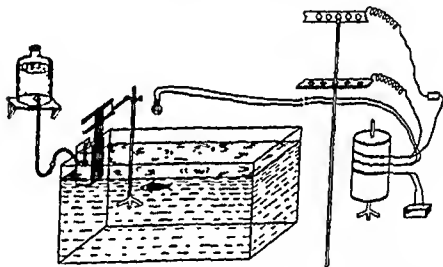


FIGURE 6 Apparatus for recording conditioned motor reflexes of fish (swimming towards the feeding box) (Prasolova 1953)

The only quantitative indicator of conditioned reflex activity in fish in experiments carried out by means of the conditioned alimentary motor method of reflexes is the speed of the final movement of the fish. It is clear that the time required to swim over to the bead depends on the position of the fish in relation to the bead at the moment of application of the signal. It is desirable, therefore, that this position should be as identical as possible in all experiments. It is therefore recommended to use aquaria consisting of two chambers (proposed by N. A. Chernova (Laboratory of Higher Nervous Activity of the Institute of Physiology Im. A. A. Ukhtomskii, Leningrad University)).

The aquarium is divided into two halves by an opaque partition with a hole in the center. The hole is covered with a mobile transparent curtain made from a film plate, and provided with a system of contacts which allow for the registration of the passage of the fish from one compartment into another. In the first compartment, the feeding box with the bead are placed (as described in the preceding method). The conditioned stimulus is switched on when the fish is in the second compartment. The signal is given and the fish swims over to the first compartment, jerks at the bead, eats the food and returns into the second compartment. At first, the conditioned stimulus is switched on when the fish is in the first compartment, near the bead, in order to elaborate the alimentary motor reflex (according to the principle described above). Application of conditioned stimulus is later concurrent with the passage of the fish into the second compartment. The presence of two conditions, feeding only after the application of the signal and switching on of the signals only when the fish is in the second compartment, caused the formation of a stereotypic motor reaction, i.e., the fish returns into the second compartment after eating the food.

#### METHOD OF CONDITIONED MOTOR DEFENSE REFLEXES

In spite of a number of advantages of the method of conditioned alimentary motor reflexes, this proved inadequate for studies on the features of conditioned reflexes in fish. On reinforcement with food the results of the experiments depend to a great extent on the alimentary excitability of the fish, the latter may fluctuate from day to day, and according to the season (this has been shown by studies carried out in the Laboratory of Comparative Physiology of Higher Nervous Activity). In addition, in experiments with food reinforcement one is confined to a small number of combinations which are inadequate for comprehensive studies on the nervous system of fish. It is thus recommended to study fish behavior by means of two conditioned reflex methods: conditioned alimentary and defense reflexes.

Yu. P. Frolov (1941) proposed a method of conditioned defense reflexes which has been used later by numerous investigators. For the elaboration of conditioned defense reflexes, electric shock serves as an unconditioned reinforcement. The current is transmitted by means of two electrodes to the body of the fish. One electrode is fixed for this purpose to a fin (this electrode is in the form of small tweezers), while the other electrode (in the form of a plate) is placed on the bottom of the aquarium. A thin copper wire passes from the electrode fixed to the fin to an induction coil. At a certain place the copper wire is attached to a button placed on a rubber membrane of a Marey capsule. Movements of the fish are recorded on blackened drum of a kymograph.

The experimental setup proposed by Frolov is illustrated in Figure 7. Procedure: At first, the indifference of the conditioned stimulus must be ascertained. If the conditioned stimulus used immediately brings about an orientating reaction, it should be rendered extinct by repeated applications of this stimulus without reinforcement. A conditioned reflex is then developed. The stimulus is applied, and 3-5 seconds later the electric current is switched on (it should be above threshold strength, i.e., a current which causes a clear-cut motor reaction). After several concurrent applications

of the conditioned stimulus and electric current, the fish commences to move actively on application of the conditioned stimulus alone.

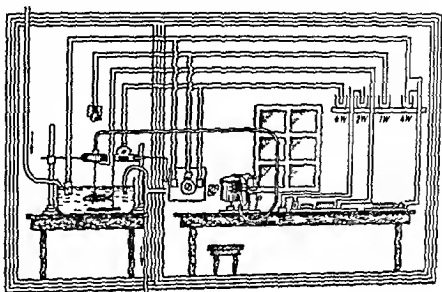


FIGURE 7 Experiment setup for studies on conditioned defense reflexes in fish (Prolov, 1941)

Various modifications of this method were primarily concerned with stimulation with electric current and the means of registration of motor defense reaction in the fish. In Figure 8 an apparatus constructed by Bull for his experiments on the development of similar reflexes is presented. A useful modification introduced by Bull consists in the electrical current passing through electrodes so as to ensure a uniform current strength throughout the electric field in the whole aquarium, and in that all recordings are done on free-swimming fish.

The drawback of the defense method of this type is that the reinforcement with electric shock is not without sequelae to the nervous system of the fish. All investigators who used this method noted that after 10-15 concurrent applications of conditioned and unconditioned stimuli, the fish cease to respond to them with adequate reaction. Some authors considered this phenomenon as a rapid extinction with reinforcement, due to the primitive state of the nervous system of fish (Karamyan, 1953). However, on the basis of studies carried out by means of other methods, it was shown that this phenomenon could be considered as the result of inadequacy of the reinforcement used. In this respect, the experimental conditions may be improved by applying unconditioned reinforcement as proposed by V.A. Sokolov and I. V. Orlov (workers of the Department of Higher Nervous Activity of the University of Leningrad) (Fig. 9).

The aquarium is divided into two identical parts by means of a partition. The partition is provided with sliding doors. On the wall of each chamber there are two electrodes. Conditioned stimuli are suspended above each of the two compartments. A conditioned stimulus is applied in the compartment in which the fish is present at that moment, three-five seconds later

an electric current is switched on which causes motor reaction of the fish. The current is applied continuously until the fish swims over to the second compartment. The fish pushes the door, thus closing the circuit, and the movement is recorded on a kymograph. If the conditioned reflex appears in the course of 5-7 seconds, no reinforcement is needed. Hence the fish may always avoid the danger of electric shock. Under these experimental conditions, unconditioned reinforcement is rarely needed. Hence, the harmful effect of electric current on the nervous system of the fish is greatly diminished.

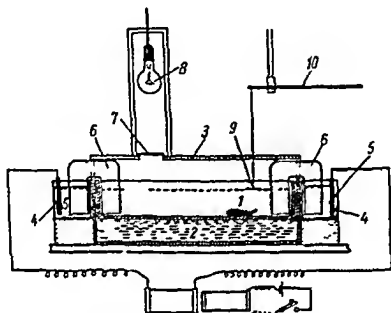


FIGURE 8 Apparatus for studies on conditioned visual reflexes in fish. Unconditioned stimulus—electric shock (Bull, 1936)

1—Fish *Blennius* in a small glass trough, 2—soundproof insulation, 3—box-screen, 4—zinc electrodes, 5—glass jars with saturated zinc sulfate solutions, 6—bridges containing agar-agar and sea water conducting current into the trough where the fish is placed, 7—slit through which the light signal is given, 8—source of light, 9—vaselin-coated coverglass for producing high surface tension the coverglass floats on the water surface and is fixed by means of waxed thread to the short arm of a sensitive lever (10) which keeps the thread stretched (in the resting state it is horizontal to the water surface), 10—arm of sensitive lever.

Recently, the workers of the Laboratory of Comparative Physiology of Higher Nervous Activity of the Institute of Physiology im. I. P. Pavlov of the Academy of Sciences of the USSR have developed a new method of conditioned defense reflexes, in which mechanical stimulation of the fish serves as an unconditioned reinforcement. The general view of the aquarium used in these experiments as well as details of the apparatus are presented in Figures 10-13.

An aquarium of plexiglass is preferable (it can easily be glued with chloroform and the various notches and grooves needed are easily made).

For fish 10-20 cm long an aquarium 50×20×25 cm in size is adequate. For the experiment, the aquarium is divided into two compartments by placing an opaque partition into the grooves (Fig. 12) (a Duralumin partition is preferred). The opaque partition is provided with transparent doors. The doors should close and open easily by means of a screw placed above the partition. They can be closed and opened simultaneously or alternately. The walls of each half of the aquarium have grooves and notches for combs which serve for mechanical stimulation of the fish. The combs are prepared from brass with nickel coated tubes fixed on a plexiglass pivot (with a small lock at the end) (Fig. 13). When in the locked position the comb lies parallel to the water surface. By opening the lock (by hand) the combs may be rotated around their axis and the investigator taps the fish slightly. The tubes should not be off the walls and bottom of the aquarium. The aquarium's corners should be cut diagonally with plates so that the fish cannot find shelter from shocks in the corner (Fig. 10).



FIGURE 9. Aquarium consisting of two chambers used for studies on conditioned defense reflexes (Sokolov 1955).

1—door in partition 2—illumination source 3-4 5-6 electrodes.

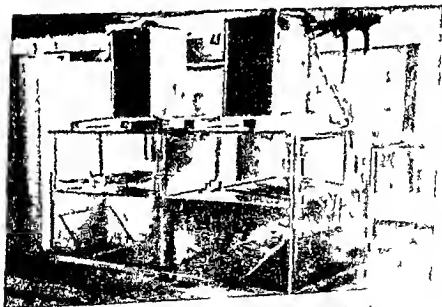


FIGURE 10. General view of aquarium for experiments on defense reflexes in which a mechanical stimulus serves as unconditioned reinforcement.



FIGURE 11 The same, at moment of application of unconditioned reinforcement



FIGURE 12. Partition with doors and screws for opening and closing doors

Conditioned light stimuli are produced by electrical bulbs suspended over each compartment of aquarium. Experimental procedure. First, the unconditioned reaction of the fish to light is studied. For this purpose, light is switched on alternately in each of the two compartments for 30 minutes, and the time spent by the fish in the dark and in the light is recorded. If the fish prefers staying in the light, a defense reflex should be elaborated, i.e., the fish should be trained to avoid light. This is achieved in the following way. Light is switched on in the compartment with the fish, and the latter is forced to swim into the dark part of the aquarium by touching it. If, on the contrary, the fish spends most of its time in the dark, light should be switched on in both compartments of the aquarium for developing conditioned reflexes. Light in the compartment with the fish is then switched off, and the fish is forced into the light compartment. A conditioned stimulus should be applied for no longer than 10-15 seconds. Unconditioned reinforcement is applied towards the 3rd-5th second. After the reflex has already been elaborated, application of reinforcement should be postponed for 10 seconds, and used only when the conditioned reaction is delayed. It was shown that per 100 conditioned stimuli in one experiment, not more than 5-10 reinforcements had to be applied. The interval between conditioned stimuli should be 30 seconds and more. Intervals of 2 minutes are preferred. In the course of developing differentiation to light of a different color, it is best to use a rhythmical stereotype of stimuli (as for the development of conditioned alimentary reflexes). Differentiation may be manifested in the fact that the fish would not swim into the second compartment on switching on the light of the other color. Using this method one is able to apply 50-100 and more conditioned stimuli in the experiment, which results in a considerable shortening of the time needed for the development of conditioned reflexes and does not cause pathological disturbances of higher nervous activity in the fish.

N. A. Chernova suggested a method for developing conditioned defense reflexes by using a different type of unconditioned reinforcement. For the experiments, an aquarium consisting of two chambers, similar to that used in experiments for studies on alimentary motor conditioned reflexes, is used. Unconditioned reinforcement causing a motor defense reaction in the fish swimming from one compartment into another compartment, may be a chemical substance (such as  $\text{CO}_2$ ,  $\text{H}_2\text{S}$  or  $\text{O}_2$ ) or a rapid withdrawal of water from the compartment. Because of the two compartments it is possible to change the chemical composition of the water in only one of them, while the fish is given the opportunity to leave the inadequate medium for an adequate one. The curtain which closes the opening in the partition precludes mixing of water of one compartment with that of the other. After the fish have left the compartment filled with harmful chemical substances, water in this compartment is changed completely through a plug in the bottom of the compartment. Since the opening in the partition is 4-5 cm above the bottom of the aquarium, the fish in the compartment with the adequate medium never suffers from lack of water. Thus the fish is always in water, and is given the opportunity to escape from the compartment with the harmful medium.

A good example of closure of conditioned links in fish is the development of a conditioned respiratory defense reflex. Carbonate solution causing a defense reaction in fish may be used as an unconditioned stimulus. This reaction is manifested in a decrease in the rate of respiratory movements and in lowering of their amplitude. The difficulty encountered in experiments



of this type is the dosage and rapid administration of unconditioned reinforcement, as well as the registration of respiratory movements in the fish. In what follows the method of study of conditioned respiratory reflexes in fish developed by N. A. Chernova is described. For recording respiratory movements, N. A. Chernova proposed to use a special device illustrated in Figure 14. The fish is fixed with the aid of an elastic rubber net suspended on threads stretching to the base of the device. On the head of the fish there is a transverse rubber band fixed in the middle to the device, while its free ends are connected by means of threads to a lever of a myograph. The rubber band surrounds the fish gills and moves together with them during respiration, and so the respiratory movements are conveyed to a kymograph. For the experiments, the entire device together with the fish fixed in it is placed in a vessel filled with water and connected to two adjacent vessels, one filled with carbonate solution and the other with water. The bottom of the vessel containing the fish is provided with a plug to remove water from the vessel. 1 l of  $\text{CO}_2$  in 1,000 ml of water and poured into 800 ml of ordinary water serves as unconditioned stimulus.  $\text{CO}_2$  solution is poured into the vessel with the fish several seconds after beginning of action of the conditioned stimulus. After 30 seconds of action of the unconditioned stimulus, the water in the vessel is changed.  $\text{CO}_2$  should be applied once per experiment. It was shown that a conditioned reflex is formed already after one combined action of the conditioned stimulus with such a reinforcement.

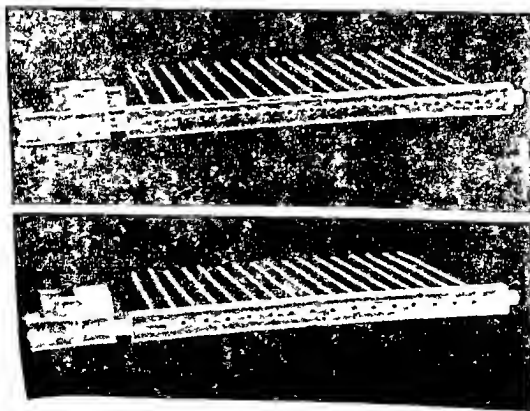


FIGURE 13 Combs with opened (top) and closed (bottom) locks

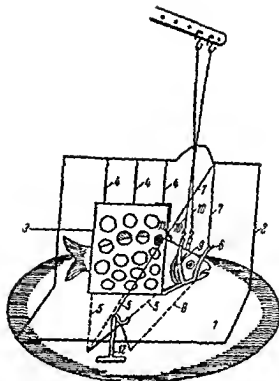


FIGURE 14 Fish in the stand (Chernovs 1953)

1—base of the stand 2—wire frame of stand 3—rubber net for immobilization of body of fish, 4—threads connecting net to wire frame 5—threads to permit movement of the net; 6—rubber muzzle 7 and 8—threads to fasten muzzle 9—recorder of respiratory movements 10—threads which connect the respiration recorder with the kymograph 11—clip to fasten the rubber net behind head of fish 12—column to wind up the fastening threads

The common feature of all methods used for the study of conditioned reflexes in fish is only the principle of conditioned reflexes and the procedure of elaboration of conditioned reflexes. Studies of conditioned reflexes are by no means exhausted by the methods described above. In each case the investigator should use his ingenuity and modify the available methods to suit his purpose and aims.

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## STUDIES ON VISION IN FISH

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### METHODS BASED ON OCULOMOTOR REACTIONS

The oculomotor reaction of fish is a variety of oculokinetic nystagmus (involuntary movements of the eye in following moving objects). The oculomotor reaction is manifest in movement of the whole fish after objects moving in the field of its vision. The biological principle of this reaction consists of adaptation of the fish to visual orientation according to motionless reference points and in sholas according to moving fish. Grundfest (1931), Wolf and Zerraha-Wolf (1936), Gaffron (1934), V. R. Protasov and K. V. Golubtsov (1960), V. R. Protasov and Yu. P. Altukhov (1960) and other scientists studied fish vision according to this reaction.

The method is as follows: the fish is placed in a cylindrical glass vessel surrounded from the outside by a rotating cylindrical screen consisting of alternating vertical bands. Spectral light (of a desired wave length and energy) is projected onto the screen from a spectrometer. For a uniform illumination of the screen, the vessel with the latter is placed on a glass table. Around the screen a truncated alabaster cone is placed. Semitransparent paper is pasted to the bottom of the vessel, the beam of light directed through the top of the table is reflected from the inner walls of the truncated cone, passes through slits between the screen bands, and falls on the vessel containing the fish (Fig. 1).

The screen rotates with a speed of 1 cm/sec. At a sufficient light intensity the fish begin to swim in the direction of the screen. A change in the direction of rotation of the screen results in a change in the direction of fish movement. If the intensity of light is diminished below the threshold of visibility, the fish cease to follow the movement of the screen (i. e. they cease to see it). The threshold of light intensity sufficient to maintain oculomotor reaction differs, depending on the width of screen bands, the interval between the bands, the speed of rotation of the screen, and the wavelength of the light. Changing the light spectrum, under otherwise similar conditions, the minimal perceptible light intensity at each length at the light wave is calculated, and the visibility curve is plotted. By determining the visibility curve in fish adapted to light and to dark, one is able to ascertain the characteristics of changes in spectrum sensitivity of vision in daytime and in twilight.

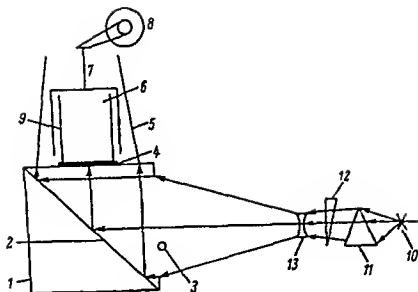


FIGURE 1. Apparatus for studies on vision in fish by means of oculomotor reaction

1-table; 2-mirror, 3-hole in table, 4-opaque glass for dispersing straight rays, 5-truncated cone (from alabaster) for uniform illumination of aquarium, 6-round aquarium, 7-cylinder supporting the screen in suspended state, 8-motor revolving the screen, 9-screen, 10-source of light, 11-tribedral prism for obtaining different regions of spectrum, 12-knob for regulating intensity of illumination, 13-lens.

By means of this method Wolf and Zerraha-Wolf (1936) determined the critical frequency of flicker at various light intensities. Wavelength, light intensity, width of screen bands and intervals between them remained unchanged. The speed of rotation of the screen and illumination varied. The screen was set in motion by means of a motor the number of revolutions of which could be regulated with the aid of a rheostat. A correlation between the intensity of light and frequency of flicker at which the images appeared continuous was noted. The cinematoscopic effect of fish vision was established in the same way by Gaffron (1934).

#### METHODS BASED ON ELIMINATION OF ACTION CURRENTS

Among methods for studies on vision of fish, methods based on action currents are of great interest, since they aid in revealing the main aspects of fish vision: perceptible spectrum, adaptation to light, color differentiation, differentiation of illumination etc. Biocurrents appear in the eye retina and in the optic nerves when light shines on them. Action currents of two types are known: retinogram and neurogram currents. By placing active electrodes on the cornea and eye fundus, biocurrents appear when light falls on the eye. These currents are characterized by oscillation frequency of the potential of the order of 50 cps. Diagrams of electrical changes in the retina following stimulation are called electro-retinograms. In Fig. 2 a retinogram of fish has been given by Day (1915). By placing active electrodes on the nerve or on the retina, electric currents of higher frequency of oscillation of potential are obtained. These biocurrents are called

neurograms (Fig. 2, 2). Neurograms consist of "action currents" of the retina reflecting signals transmitted to the brain from the eye (Adrian and Matthews, 1927). A neurogram of a cod (*Gadus morhua morhua* L.) is presented in Fig. 2, 2. When light and darkness alternate, or when changes in light intensity occur, these are accompanied by changes in the frequency of oscillation and the magnitude of potential of the "action current" (Fig. 2, 2).

Changes in neurograms thus reflect the changes in perception of light stimuli by the eye. Hence, comparison of changes in the neurograms with those of light stimuli acting on the eye (known to the investigator) is the basis of electrophysiological studies on vision in fish.

1. Comparative method. This method is based on a comparison of periodic changes in the action of light and darkness on the eye with changes in the retinograms or neurograms. This method has been used by various authors for the determination of the capacity of eyes to perceive individual light flashes (studies on the critical frequency of flicker merging). The critical frequency of flicker is one at which the eye is able to perceive continuous motion. By using this method, Day (1915) showed that tenches and pike are capable of perceiving 25 individual light impulses per second as separate. Svetichin (1955) showed that in the cones of the eye of perch and breams the critical frequency of flicker is 55/second.

2. Threshold method. This has been developed in detail by Granit (1947). It allows for the determination of eye sensitivity to light of various wavelengths and at various states of adaptation to light. This method is based on finding the minimum energy of light at all wavelengths (threshold stimulus) causing the appearance of action currents in the retina or optic nerve. Using this method, Granit established the relative spectrum sensitivity in the eyes of carps, eels and tenches adapted to light and to darkness. A Purkinje\* effect has been established in these fish, indicating the presence in retina of "daylight" and "twilight" receptors.

3. Colorimetric method developed by M. M. Bongard and M. S. Smirnov (Bongard, 1955) who constructed a special colorimeter for studies on color vision in animals. This method is based on a comparison of bioelectric activity of the optic nerve (or retina) with the action of changes in the field of vision of the colorimeter. If the eye does not "see" the border between "fields of vision" of the colorimeter presented to the fish the "action currents" do not appear in the optic nerve (retina), and vice versa. Proceeding from this, the author gave the following definition of color vision. The eye does not differentiate the given color if a ratio between two colors acting alternately can be found at which no "action currents" occur.

If, on the other hand, at all ratios between the intensities of two colors, "action currents" do occur, it is assumed that the eye of the animal differentiates these colors.

The colorimetric method allows one to study the capacity of the eye to differentiate colors and light intensity. In contrast to the threshold method, this method allows one to study the reception of light by the eye at above-threshold intensities (bright illumination).

\* "Purkinje phenomenon" - change (occurring in periphery) in the relative brightness of color with illumination and changes in spectrum sensitivity in eyes adapted to light. For details see S. Krasov (1927)

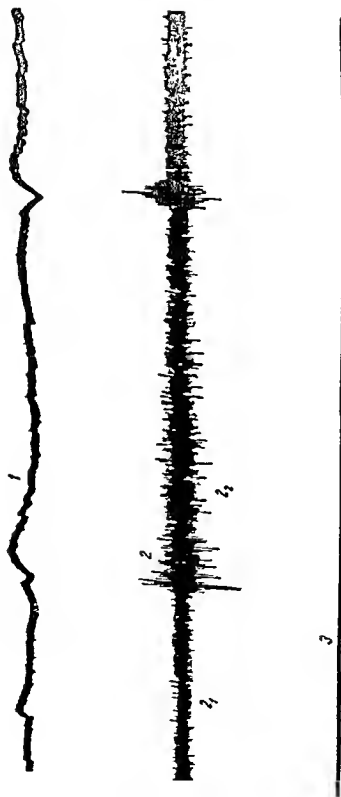


FIGURE 2. Electric currents in eyes of cod (*Gadus morhua morhua* L.)  
 1 retina; 2-neurogram; 3-application of stimulus; 2<sub>1</sub>-rate of electric activity of retina before application of the stimulus; 2<sub>2</sub>-rate of electric activity during action of the stimulus and some time after. Surges can be seen on the neurogram.

Action potentials must be studied on living fish (retina sections are difficult to prepare and cannot survive the duration of the experiment)

To obtain retina preparation ("surviving retina preparation" according to the terminology of Svetikhin, 1955) the living fish is subjected to eye enucleation followed by careful removal of the lens, vitreous humour and sclera from the retina (Fig. 3). The retina preparation is illustrated schematically only in Fig. 3. The "retina preparation" of the eye of cartilaginous fish is viable for several hours. The living fish must be operated on. The optic nerve or retina is exposed (depending on whether a retinogram or neurogram is to be obtained, i. e. on the initiating point of the action current). Various segments of the optic nerve must be exposed, depending on the anatomical features of the various species of fish. In some fish (such as anchovy) the optic nerve can be approached only transcranially. In other fish (plaice, burbot and sheathfish) the optic nerve is very accessible.

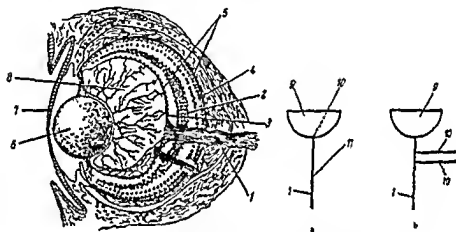


FIGURE 3. Location of electrodes for tapping of action currents from the retina and optic nerve in fish. Left figure - anatomy of eye of bony fish (cross-section through the middle of the eye) right figure - placing of electrodes in methods of tapping of action currents in eye of fish (a-bipolar, from retina, b-bipolar, from optic nerve)

1-optic nerve, 2-bipolar cells of retina; 3-ganglion cell layer, 4-layer of cones and rods, 5-retina, 6-lens; 7-cornea; 8-vitreous humour; 9-dissected eye, 10-active electrode; 11-earthed electrode

The fish is placed on an operating table in a tub. For respiration water from a reservoir passes through a tube and into the mouth of the fish, rinses the oral cavity and gills and passes through the gill slits into the tub and outside through a plug.

Normal movement of gill covers indicates normal respiration. When movement of the gill covers increases, the rate of water flow should be increased. The fish to be operated on is fixed on the operating table, the form and size of which depends on the size of the fish. Curare, diploclin and other drugs must be given before the operation to immobilize the eye. In operations on the eye, diploclin is mostly used. It has been shown that injections



of 0.5% aqueous solution of diplacin are satisfactory. The dose should be chosen by experiment.\* Depending on the dose, various muscles are rendered inactive (muscles of the body, fins, head and branchiostegal apparatus). The dose should be such as not to paralyze the respiratory movements, while ensuring maximum immobility.

For the operation the usual surgical instruments are used (scissors, pincers, scalpel). The operation should be carried out under a binocular microscope (because of the limited operation field) under bright illumination (X100 magnification should be used). To avoid heating of the operation field by the light beam of the microscope, heat filters absorbing infra-red rays should be used.

In studies on action currents from the optic nerve the sheath of the latter should be exposed. In studies on action currents from the retina, the anterior chamber of the eye should be removed. To this end, the sclera is exposed and removed, the muscles of the lens are cut and the lens enucleated. The vitreous humour is then carefully removed. The integrity of the retinal blood vessels should be carefully checked.

The operated fish together with the operating table is transferred to a screened chamber of the electrophysiological apparatus. The active electrode is inserted in place in the eyeball under a binocular microscope. The electrode is best placed on the blind spot on the retina. The electrodes used are usually prepared from platinum wire of varying cross-section (depending on electrode location) sealed in glass\*\*.

There are two types of electrodes: 1) electrodes with a "working tip" and 2) "hooked" electrodes (Fig. 4). In electrodes with a "working tip" the platinum wire terminates at the same level as the glass case. These electrodes guard from any shunting effects of the surrounding fluids and may be used for studies on action currents from the optic nerve and retina cells by placing them directly on the surface of the nerve or retina cells. In the "hooked" electrodes, the platinum wire protrudes from the glass tube in the form of a hook. In that case the optic nerve should be hooked onto the electrode. The electrodes are fixed to a tripod inside the screening chamber with the aid of mobile hinges.

The correct location of electrodes is checked by the electric response to periodic light flashes which are directed to the eye. The preliminary study is thus completed.

The electrophysiological setup for studies on vision in fish may vary depending on the aims of the investigation. They all consist of three main parts: 1) source of light - to create light impulses of required quality and intensity, 2) system to record biocurrents; and 3) screening chamber.

1. Light transducer. Construction of this unit may vary, depending on the aim of the investigation. In general it consists of: 1) source of light; 2) light transformers - optic apparatuses; 3) light-measuring devices. The source of light may vary; whatever the source, the following conditions must be fulfilled: it should have a high light intensity, all wavelengths of the spectrum must be available; they must be easy to handle. Cinema projection lamps of the K-22† type as well as projectors fulfil all these conditions.

\* Bottom-dwelling and sluggish fish require a higher dose than pelagic and active fish.

\*\* For the preparation of electrodes, see O. Strong, 1947.

† For standard designations, see "Spravochny knizh po svetotekhnike" (Manual of Illumination Engineering), 1957.

The light-measuring device consists of a light receiver and a sensitive galvanometer graduated in light units (lux). Selenium photoelements usually serve as receivers of light. Selenium elements should produce a high photocurrent (500 amperes per lumen) and their spectrum sensitivity should be as close to that of the human eye as possible.

Various galvanometers may be used. Galvanometers sensitive to  $10^{-8}$  to  $10^{-9}$  amperes acting together with selenium photoelements are sufficient to measure light of 1/100 to 1/1000 of lux. To measure light of lower intensity, special apparatuses are required (they can also be measured by calculations — the energy absorbed by neutral light filter placed between the eye and the photoelement is calculated).

Light used in these experiments may vary. Various electrophysiological apparatuses are suitable according to the light used. The main types are those which make use of a light interrupter, monochromator and Borgard and Smirnov colorimeter.

**Light interrupter.** Light interrupters are used in studies aimed at estimating the capacity of the eye to perceive individual flashes (light impulses). The light interrupter is a disk with holes around its circumference, which may be set in motion with the aid of a motor. The number of rotations per minute may be regulated with the aid of a rheostat and measured with the aid of a tachometer. The disk is placed perpendicular to

the light which falls on the eye. By means of rotation, the disk allows light to enter or obstructs its passage to the eye (Fig. 5).

**Monochromator.** This is a spectrum apparatus which allows to resolve the desired wavelength of light from the beam of "white light", and to regulate it according to its energy. The prismatic monochromator (Fig. 6) consists of a prism and entry and exit collimator, entry and exit slits. A beam of "white light" enters the slit of the collimator, and the prism reflects it into a continuous spectrum. By turning the prism a monochromatic ray (segment of spectrum close to the wavelength of the given light) is directed to the eye through the entry slit.

Monochromators used for electro-physiological investigations of vision should have a high light power of their optics, and a high dispersion of their prism. Monochromators of the UM-2 and MS-Sh type are quite satisfactory (for details of the production of monochromators in the USSR, see the book by A. Toporets, 1955).

With the aid of monochromators, the spectral sensitivity of the eye in various states of adaptation to light may be determined. The monochromator may be replaced by a set of interferential light filters of the visible spectrum which allow one to use spectrum segments close to monochromatic rays.

\* A single-tube amplifier is insufficient for vacuum type antimony - cassium photoelectric cells feeding only small photocurrents to the galvanometer. Description and use of photoelectric cells may be found in the book by N. Chechik (1955).

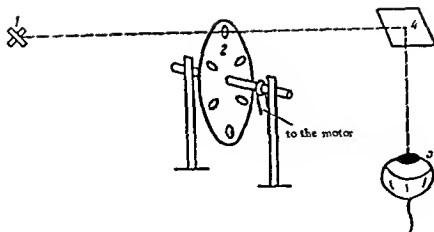


FIGURE 5. Light interrupter

1-source of light, 2-disk with holes, 3-eye; 4-mirror.

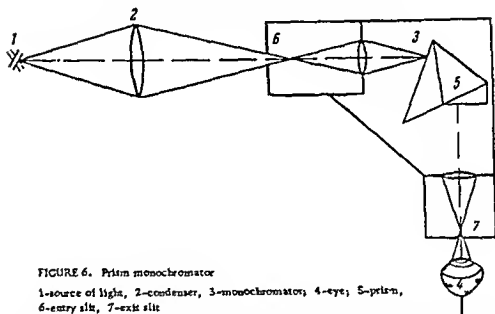


FIGURE 6. Prism monochromator

1-source of light, 2-condenser, 3-monochromator, 4-eye, 5-prism, 6-entry slit, 7-exit slit

The Bongard and Smirnov colorimeter (Fig. 7) is used for electrophysiological studies on color vision in animals. The apparatus enables one to transmit two radiations to the eye of the animal studied, one of which may be a mixture of light of two wavelengths. The colorimeter is a monochromator with several entry slits. In the lower part of the monochromator slit (Fig. 7 on the right) plate 8 with a lower vertical slit (9) may move horizontally. As a result, the wavelength of light emerging from the monochromator is altered. Plate 10 with two or three vertical immobile slits (11, 12) (the so-called basic slits) is fixed in the upper half of the entry slit of the colorimeter.

Passing through basic slits, the light emerging from the colorimeter consists of a mixture of monochromatic rays. The energy of light passing through the basic slits is regulated with the aid of screws 13 and 14 which close or open these slits. The energy of light passing through the lower slit is regulated by means of a light wedge 15 placed in the grooves of the lower part of the entry slit.

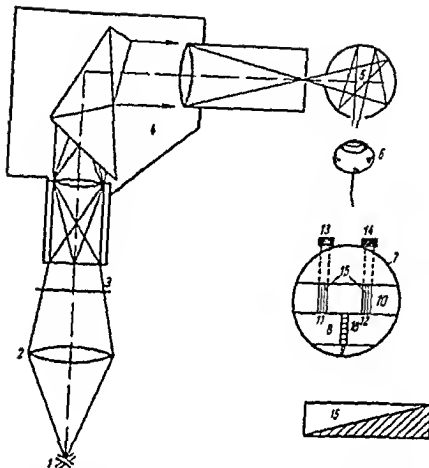


FIGURE 7. Colorimeter constructed by Bongard and Smirnov, on the left - general view, on the right - entry slit of colorimeter

1-source of light; 2-condenser; 3-polaroid; 4-colorimeter; 5-disperser; 6-eyes; 7-entry slit; 8-lower mobile plate with slit 9, 10-upper immobile plate with slits 11 and 12; 13-14-screws for regulating light passage through slits 11 and 12; 15-mechanical light wedge; 16-polaroid plate on slits at entry to colorimeter.

To replace one wavelength by another (light passing through upper and lower slits) the polaroid system 16 is used. The upper slits are closed with the aid of the polaroid plate oriented reciprocally perpendicular in relation to the polaroid film of the lower slit. Rotating polaroid 3 is placed before the entry slit. Due to the reciprocally perpendicular orientation of the polaroid plate in the upper and lower slits, rotation of the mobile polaroid causes the light to pass either through the upper or lower slit. A change in wavelength is thus provided. To produce a uniform stream of light to the eye, the beam of light emerging from the monochromator passes through a disperser (5), a hollow sphere with its inner surface painted white.

This colorimeter is used in studies on the capacity of the eye to distinguish colors and light intensity.

2. System of registration of "action currents" in the eyes of fish. Systems of registration of nerve "action currents" used in

electrophysiological experiments have been described in detail by Gulyaev and Zhukov (1948) and Shminke (1956). The registration device consists of amplifiers and recording devices. "Action currents" originating in the retina and optic nerves of fish are represented by weak electric potentials (to 100 microvolts). The frequency characteristic of "action currents" presents a curve of from 50,000 to 2,000 impulses per second, with a peak at 600 - 800 impulses per second.

Owing to the biological properties of tissues of fish, the electric resistance at points at which the "action currents" are measured is enormous (of the order of 100,000 - 1,000,000 ohms).

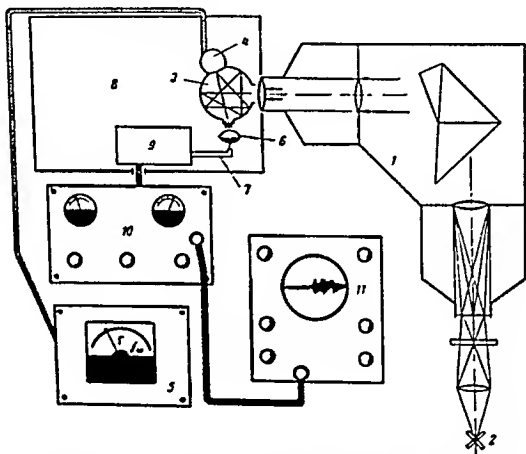


FIGURE 8. Portable electrophysiological apparatus for studies on fish vision

1-colorimeter-monochromator; 2-source of light; 3-disperser (Ulbricht sphere), 4-photoelement, 5-galvanometer, 6-eye of fish; 7-electrodes, 8-screening chamber, 9-preliminary amplifier, 10-terminal amplifier, 11-cathodic oscillograph.

These electric parameters show that to amplify "action currents", amplifiers of low sound frequency within the range of micro-currents, high input resistance (one mega ohm) and high amplifying coefficient ( $K = 10^6$ ) can be used.

We have used cathode-ray oscillographs of the EO-6 type, six stage amplifier of alternating current of low frequency, and an M-24 microammeter.\*

\* For detailed description of recording devices see manuals on radioengineering (Bonch-Bruyevich, 1950, Remes and Khiz, 1947).

For detailed studies on "action currents" we have recorded them on a ferro-magnetic tape of a tape-recorder (the tape recorder was placed at the outlet of the amplifier) The best tape recorder which recorded undistorted "action currents" proved to be the MAG-8 tape recorder having a uniform pass band for the frequency range of "action currents" of the retina

3. Screening chamber This is used for screening the fish from electromagnetic disturbances during studies on "action currents" The screening chamber is a wooden box lined with tin plate, and earthed The chamber contains the following parts of the electrophysiological device a) the preliminary stage of the amplifier, b) flow system\*, c) exit collimator of the colorimeter with light disperser, d) the photoelement for measuring light falling on the eye, e) electrodes

Before placing the fish into the screening box for the experiment, the entire apparatus should be checked At first the function of the amplifier is checked If it is in order, disturbances appear on the oscillograph screen when the screening chamber is open (usually 50 cps from the line of alternating current) When the screening chamber is closed the disturbances disappear, and the oscillograph screen shows a horizontal band 0.5 - 1.0 cm in width (noise of electronic lamps of the amplifier and the noise indicating opening of entry point). The cleanliness of the electrodes is then checked For this purpose the electrodes are switched on to the amplifier, with their tips placed on moistened cotton wool. If the electrodes are clean the oscillograph screen shows no disturbances The horizontal band (noise) on the oscillograph screen narrows in the process The electrodes are then rinsed in alcohol

Monochromatism of the light emerging from the monochromator can be checked with the aid of a pocket spectroscope Monochromatism of light is followed according to the scale of the spectroscope If the light consists of waves of varying length, the exit slit should be narrowed

When a colorimeter is used, it should be ascertained that the wavelengths can be changed evenly. smooth operation of the polaroid plate on the upper and lower slits at the entry to the monochromator should be checked Correct position of the polaroid plate on slits of the colorimeter is confirmed with the aid of a galvanometer and photoelement. If the plate on the slits is orientated correctly and the amount of light passing through the upper slit is identical with that passing through the lower slit then an identical amount of energy should pass through the exit slit at all positions of the mobile polaroid (the pointer of the galvanometer should remain immobile), otherwise the plates on the colorimeter slits should be reglued

Dispersion of light is then checked Uniform dispersion of light passing from the sphere is checked with the aid of the galvanometer connected to the photoelement. If the beam of light passing from the disperser is not uniform, the former should be replaced\*\*.

The action of the photoelement and galvanometer should also be checked If the amplifier is switched on and disturbances appear on the screen of the oscillograph, the galvanometer should be earthed

Having checked the work of the colorimeter, the fish is placed in the screening chamber and the experiment may be commenced The experiment starts with adaptation of the eye to the required light intensity

\* The structure of the flow system of the screening chamber is the same as that of the flow system of the operation table

\*\* For the construction and theory of dispersers, see the book by F. Tikhonov, 1934

We have constructed the portable apparatus colorimeter (Fig 8) for studies on edible marketable fish carried out under field conditions.

The experiments on vision of fish may be carried out with the aid of bio-currents under any conditions, if an electric current can be provided. This method yields rapid results and permits one to carry out experiments on fish which do not react well to conditions in aquariums.

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## METHODS OF STUDY OF HEARING IN FISH

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Parker (1903) and Bigelow (1904) were the first to show that hearing in fish is associated with the labyrinth. This has subsequently been confirmed by other workers.

The labyrinth of bony fish consists of an upper and lower part. The upper part comprises the utricle with three semicircular canals arranged in three reciprocally perpendicular planes. The upper part of the labyrinth whose function is to maintain equilibrium is connected by a short canal with the lower part which consists of an oral sac (sacculus); one of the walls of the oral sac forms the lagena (a considerable swelling). (Fig. 1). Sacculus and lagena were shown to be endowed with an auditory function in carp (Frisch and Stetter, 1932).

The anatomy of the ear differs in various fish species, in some species auxiliary auditory organs are present.

In studies on the ability of fish to perceive sounds and on their sound sensitivity etc., the behavior of fish in response to sound stimuli is tested (Zenneck, 1903, Parker, 1903, 1909, Bigelow, 1904, Haempei, 1911 and others). This method is used at present for studies on the reaction of edible fish to sound. The reaction of fish to sound varies, it may be manifest in turning towards the source of sound, in escape from the sound, in general increase in motor activity, increased fin movements, change in body position or change in the frequency of respiratory movements, and others. This method has only a limited use, however, because a number of fish species show no reaction to sounds (this does not mean that these fish are devoid of hearing, but it may mean that in these fish the sound applied is of no biological significance), while in others repeated application of sound leads to extinction of reactions to the sound applied.

The method of conditioned reflexes is widely used in studies on hearing in fish (Froloff, 1925, 1928, Bull, 1928, 1930, Stetter, 1929, Frisch und Stetter, 1932, Boutteville, 1935, Disselhorst, 1938, Wolfahrt, 1939, Dijkgraaf and Verheijen, 1949, Dijkgraaf, 1950, Kleerecooper and Changon, 1954, Malyukina, 1955, 1958, 1960). This method allows one to study a number of important features of hearing in fish.

The choice of method (allimentary or defense) is determined by the ecological features of the fish studied.

Sound is used as a conditioned stimulus. This is generated with the aid of a sound generator and is communicated to the emitter. Since sound loses

a considerable part of its energy on passing from air into water, the source of sound should be placed under water. A single telephone or transducer may be used to produce sound. To create sounds of high frequency, magnetostrictive piezoelectric emanators may be used. To protect the source of sound from being moistened by water the former is placed in a plexiglas or metal cover. It may also be covered with laquer or glue.

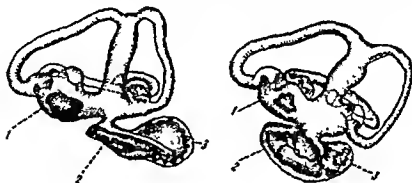


FIGURE 1 Labyrinth in minnow (left) and in trout (right). After Frisch, 1936

1-utricle; 2-saccule; 3-lagena

To avoid side vibrations, the source of sound should not be fixed on the walls of the aquarium.

Prior to elaborating conditioned reflexes, the fish should be adapted to the experimental conditions (to the source of sound placed in water, to the electrodes when the electric-defense method is used, and soon). In some fish, especially in marine shoal fish which become excited when placed in isolation, this stage can last for a considerable time.

The experiments are usually carried out in aquariums where the fish live.

Conditioned reflexes are developed according to the conventional stereotype. In some fish they appear after several combined applications of unconditioned and conditioned stimuli, in other fish the conditioned reflexes are developed with greater difficulty. Other fish still are incapable of developing conditioned reflexes to sound (Bull, 1928, 1930, Moorehouse, 1933, Frisch, 1936, Farkas, 1936, Disselhorst, 1938, Bara, 1955, Malyukina, 1960).

Fishes most frequently used in experiments (carp, crucian carp, brown bullhead, goldfish, minnows) develop clear-cut and stable conditioned reflexes to sound.

In the overwhelming majority of fish the frequency and dynamic range of sound perception are unknown. Hence, fish in which the organ of hearing is removed serve as control to ascertain whether the conditioned reflex is linked with the sound analyser, and is not due to some other reception (skin reception, for example).

An operation involving the section of the acoustic nerve is not satisfactory, since it impairs not only the hearing but also the equilibrium of the animal. Frisch and Stetter (1932) developed a delicate and accurate method for removal of the organ of hearing in the minnow (*Phoxinus phoxinus*). The organ of hearing in carps (sacculus and lagena of the labyrinth) is located below the brainstem, immediately below the lobi vagi (Fig. 2).

They lie in the hollow of the cranium, separated from the brain by a thin bony partition. This location of this part of the labyrinth prevents any surgical approach from above. It can be approached only from the side, through the wall of the bony chamber where the inner ear is located.

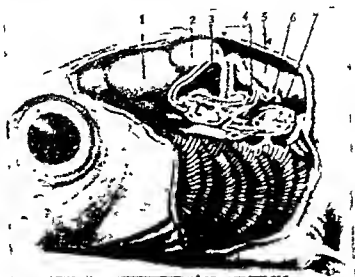


FIGURE 2. Labyrinth in minnow skull (Frisch and Stetter, 1932)

1-optic tectum, 2-body of cerebellum, 3-utricle, 4-sacculus,  
5-lobi vagi, 6-vagus nerve, 7-lagena



FIGURE 3. Removal of organ of hearing (after Frisch and Stetter, 1932)

1-border between prooticum and exoccipitale, 2-glos-  
sopharyngeal nerve, 3-vagus nerve

The fish is anesthetized by placing it in a 0.5% solution of urethane, it is then removed from the urethane solution and fixed to an operating table. The fish head is placed in a tube through which passes water to sprinkle the gills and a 0.25% solution of urethane for anesthesia. The operation is carried out under a

binocular microscope. The gill cover is pulled forward and upward with the aid of a thin hook (Fig. 3). A thread stretching from the hook is placed through a pulley, and with the aid of weight tied to the end of the thread the gill cover is maintained in the desired position. Muscles stretching to the third and fourth gill arches are dissected longitudinally (with the aid of a needle) and pulled aside. A powerful muscular band located caudally is pulled aside with the aid of a blunt hook and weight. This muscle should not be damaged, otherwise swallowing is impaired. Then the tissues are pulled slightly down and aside, exposing the border between prooticum, exoccipitale and basioccipitale (Fig. 3). A small opening is drilled behind the prooticum exactly on the border between exoccipitale and basioccipitale. This should be done so as not to damage adjacent blood vessels. The sacculus is immediately below the hole. The latter is hooked by a thin hook, lifted a little towards the opening and pulled out carefully with the aid of pincers. The clubshaped otolith of the sacculus is also lifted with the aid of a hook toward the opening, and pulled out with pincers. Frequently the lagena with its otolith cannot be removed simultaneously. To do this after having removed the sacculus, a thin hook is inserted into the opening and directed caudally. The lagena with its otolith is hooked, lifted toward the opening and pulled out. The operation on the second labyrinth is carried out some time after the first operation.

In some fish which recover from the operation, disturbances in equilibrium may appear several days after the operation. This is connected with inflammation of the utricle. Such fish are unsuitable for the experiment.

When the fish operated on begin to swim and take food, the established conditioned reflexes may be tested, or new reflexes may be developed.

At the end of the experiments, complete removal of the organ of hearing is verified by means of visual control.

For studies on hearing in fish, and in particular on the mechanism of sound perception, numerous workers used the electrophysiological method.

Adrian, Craik and Sturdy (1938) killed fish (eels and pike) by decapitation, exposed their cranium and destroyed the brain at the level of the cerebellum. Part of the medulla oblongata, isolated from the spinal cord by cutting the former together with the eighth cranial nerve, were placed on a small ring-shaped electrode made from chlorine-silver wire. The second electrode, a spiral silver wire, was located on the other side of head. In other experiments, cotton woolwicks placed on the acoustic nerve near its exit from the inner ear served as an electrode. Biopotentials were conveyed to an amplifier and then to an oscillograph, where they were recorded. Speech or singing as well as pure tones from a siren applied at various distances from the ear of fish, served as stimuli.

Sound stimuli were recorded simultaneously with recording of responses of the acoustic nerve. For this purpose, a microphone was connected with the second oscillograph.

In experiments of Zotterman (1943) pikes and burbot were killed by decapitation and their heads were dissected in a sagittal direction along a median line. Branches of the acoustic nerve running to the sensory epithelium of the sacculus were isolated, and some of the nerve fibers were sectioned. Nerves running to the posterior ampoule of the labyrinth and to the sensory epithelium of the lagena were also sectioned. Biopotentials were transmitted with the aid of chlorine-silver electrodes. For studies of processes taking place in the sacculus on sound stimulation, microelectrodes inserted with the aid of a micromanipulator were used.

Biopotentials were transmitted to an amplifier and were recorded on an oscillograph. The sound of a tuning fork served as stimulus.

In more recent works of Lowenstein and Roberts (1951), Katsuki, Uchiyama and Totsuka (1954), the electrophysiological method was successfully used in studies on the mechanism of sound perception and on the sensitivity of various parts of the inner ear and even of individual cells of the auditory epithelium of fish to sound.

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## METHODS OF STUDY OF TASTE AND SMELL IN FISH

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### METHODS OF STUDY OF TASTE RECEPTORS IN FISH

The function of sense organs for taste and smell is determined by the action of stimuli dissolved in water on receptors of smell and taste. In human physiology it is accepted that receptors for the sense of smell are stimulated by gaseous substances, while those for the sense of taste on contact with dissolved substances. This is not so in fish. Morphologically and functionally, however, the sense organs of taste and smell differ from each other.

Receptors for the sense of taste in fish are located in special paired sacks, each of which opens into one (in Baltic herrings) or in two (in bony fish and ganoids) openings. Water circulates through these nostrils when the fish moves forward.

Water circulation may also be determined by special devices (Bateson, 1890, Burne, 1909, Pipping, 1927). The histological structure of smell receptors has been described by Y. A. Vinnikov and L. K. Titova in their monograph published in 1957.

Receptors for the sense of taste in fish, like those in higher vertebrates, are located in the oral cavity, but owing to the aquatic way of life of the fish, they may be located on external covers, being encountered on lips and barbels, and in some fish on the entire body (Herrick, 1903, Schneider, 1931, Dykgraaf, 1934, Sato, 1937c, Disler, 1953). These external receptors act as exteroceptors. The most morphologically specialized receptors of taste are the barbels and filamentous rays of fins in numerous fish, as well as the free fin rays in Triglidae. External receptors for the sense of taste are taste buds similar in structure to taste buds present in the mouth. In Triglidae the specialized rays of the pectoral fins contain special elongated cells which do not form group structures (Scharrer, 1935).

Receptors of the sense of smell are represented by primary sensory cells. Their innervation comes from the olfactory nerve and the primary olfactory centers are located in the fore-brain. Nostrils are also innervated by branches of the fifth cranial nerve (trigeminal), whose endings play some role in perception of pungent irritating substances (Sheldon, 1909).

Receptors for the sense of taste are represented by secondary sensory cells which are innervated by the seventh, ninth and tenth cranial nerves. Primary taste centers are located in the medulla oblongata. Innervation of taste receptors of the free fin rays of Triglidae is an exception, in that they are connected with the roots of the spinal cord.

Receptors for the sense of smell play the role of distance receptors, while the receptors for the sense of taste are contact receptors (Parker and Sheldon, 1913). These two groups of receptors may also be differentiated by the quality of stimuli which affect them (Strieck, 1924).

#### METHODS OF STUDY ON TASTE RECEPTORS IN FISH

All methods available for studies on taste sensation in fish require still further improvements (because of the difficulty in applying chemical stimuli dissolved in water). One of the methods used for studies on taste perception is based on watching the reaction of fish in response to the stimulus tested. This method enables one to evaluate the behavior of animals towards various substances, i. e. to disclose specific unconditioned or natural conditioned reflexes to various chemical stimuli.

The adequacy of stimuli used should be considered. Herrick (1903) pointed out that Nagel (1894) erred in his studies on taste perception of some fish by placing bitter, sweet, sour or salt solutions on their body, and watching their alimentary reaction. Since he failed to obtain the anticipated response to the stimulus applied, Nagel concluded that the skin of carp and sheathfish is insensitive to taste stimuli. Herrick, however, used food stimuli - meat and meat infusion - and noted the reaction of fish to stimulation of the skin.

Studies on external receptors for the sense of taste. Various organisms serving as food, meat, cotton soaked in meat infusion, pure meat infusion, meat soaked in various solutions, etc. may be used as stimuli in studies on external taste receptors. The sensitivity of lips to taste is difficult to reveal, since the stimulus may find its way into the oral cavity. However, there are several simple methods which may establish the presence of taste receptors in the epidermis of the lips or snout. These methods are especially suitable for studies on little mobile benthic fish. Meat infusion (juice) stained with methylene blue is carefully discharged from a pipet above the blinded fish. The flow of juice is watched and the catching movement of the fish is recorded (Andriyashev, 1944, 1944c). In control fish the nostrils are closed by plasticine.

The most accurate control is that making use of histological methods for the detection of taste buds.

It is more convenient to study specialized caeca bearing receptors for the sense of taste, i. e. barbels and specialized fins as well as the various segments of the body. Stimulation of barbels of the brown bullhead (*Ameiurus*) or *Gaidropsarus* with meat served on swabs or on cotton soaked in meat juice is usually followed by these catching movements (Herrick, 1903, Andriyashev, 1944a, and others). A similar reaction takes place when the barbel is stimulated by a thin stream of meat juice - this is easily done in fish whose barbel tips are far removed from the mouth. Such experiments were carried out by Herrick (1903) on brown bullhead, and by Sato (1937a, 1938) on Japanese red mullets.

To study the role of barbels and fins in search for food the bait (worms, etc.) may be dug in silt, meat may be wrapped in gauze (Sato, 1937, 1937a, 1937b, 1938) or meat may be placed between two empty shells (Herrick, 1903).



The method of preparing gauze envelopes will be described below. In control experiments, the sensation of smell must be eliminated. Methods based on giving hidden bait allow one to investigate the specific function of barbels or other organelles and specialized organs.

Differentiation of taste from tactile perception. In studies on external taste perception, cases may be encountered in which the fish (for example brown bullhead) responds with a catching movement to stimulation with clean cotton applied to the barbel. This reaction was called "tactile reflex" by Herrick (1903) in contrast to "taste reflex". If stimulation with clean cotton is repeated several times the catching reaction is extinguished, but appears when the barbel is touched by meat or by cotton soaked in filtered meat juice. According to the data of Herrick, this response is not extinguished even by repeated stimulations.

When barbels are to be stimulated by various solutions discharged from a thin pipet or squirted from a syringe, the reaction to tactile stimulation should also be extinguished, for this purpose pure water is discharged from a pipet or squirted from a syringe. This procedure should be repeated several times.

In this type of experiment, tactile stimulation cannot be completely avoided, since the tactile component is included in the taste stimulus. To clarify the role of receptors for the sense of taste in spatial distribution, Herrick carefully discharged strained meat juice from a thin pipet which, due to its greater specific gravity, sank in the water and stimulated the tip of the long barbel of the brown bullhead. This fish responded to the stimulus by a directed reaction which was somewhat delayed as compared to that noted in response to stimulation with meat.

Section of nerves which innervate the taste buds on the body and fins of fish also enables one to discriminate taste stimuli from tactile ones. Tactile sensitivity of the fish body is due to free nerve endings (from upper roots of spinal cord - Herrick, 1903, 1903a). Cutting of specialized taste nerves in loaches (Dijkgraaf, 1934) and in marine burbot (Aronov, 1959) showed that tactile perception remained, but the fins of body on the operated side ceased to respond to food stimuli by alimentary reaction, while stimulation of other surfaces the innervation of which was intact brought about an alimentary reaction.

For studies on the role of the tactile component in response to food stimulation, various objects are soaked in meat juice. In this way Herrick (1903) checked the reaction of brown bullheads to pieces of brick soaked in meat juice. As a rule the fish took this bait.

Difference in sensitivity of external and internal receptors for taste sense. Receptors for the sense of taste localized in barbels and in the oral cavity may be distinguished by their sensitivity to various substances. One method for detecting such differences consists in soaking of food in various solutions.

Bait soaked in quinine is taken by the carp into their mouth after probing it with their barbels (Wunder, 1927). Meat soaked in bitter absinthe infusion is caught by sea burbot (Andriyashev, 1944a). After checking the food by receptors present in the mouth, the fish spit the bitter food out. The reaction to sweet substances may be quite different. Sea burbot readily swallows meat soaked in sugar solution. Food treated with 5% solution of hydrochloric acid brings about an escape reaction (after the fish has touched the bait with its barbels). In a similar way Japanese red mullets recognize earthworms.

soaked in weak acetic acid. This ability is lost in fish in which the barbels are cut (Sato, 1937).

Sensitivity of fish to taste stimuli depends on the number of taste buds per unit of area (Herrick, 1903).

Electrophysiological studies on external receptors for taste sense. The fish studied should be devoid of the capacity to move, by sectioning its medulla oblongata or by puncturing it and the spinal cord (curare, diploicin and other similar drugs are now used instead). Nerves were exposed at their points of exit from the brain (Hoagland, 1932, 1932a), and the segment of the nerve branch exposed for 3-8 mm was proximally tied with a thread, sectioned distally and tied to an electrode. This procedure should be followed so that the receptor fields remain immersed in water. The nerve and the wound should not be in the water, and should be moistened with Ringer's solution. Chlorine-silver electrodes were used, these were connected through two amplifiers to an oscillograph. The circuit also included a loudspeaker connected to an additional amplifier.

Acetic acid solutions of various concentrations (from 1 to 20%), a 10% solution of hydrochloric acid, and saturated sucrose solution served as taste stimuli. Meat juice was also used. Prior to application of the chemical stimuli the action potentials of the barbels on mechanical stimulation with feathers were tested. The solutions to be tested were slowly discharged in to water near the preparation and diffused to the barbels. Impulses from taste stimulations were recorded on the oscillograph screen, but were especially easy to detect on the sound amplifier.

For studies on preference for various food in crucians, food cannot be mixed with white bread, since this fish readily eats bread. Hence meat homogenates were mixed with sand. Food mixtures were placed on special devices. A glass plate somewhat narrower than the bottom of the aquarium was divided into four equal areas by thin glass bands. Along the edges of the plate, glass bands were placed so as to form four very small chambers whose borders were invisible after filling them with sand. The fields were filled with a mixture of sand and finely ground edible organisms of various species. The weight of edible organisms comprised 1 - 5% of the weight of the sand. One field serving as control was filled with pure sand. This feeding rack was carefully immersed in the aquarium. After the ripples caused by placing the rack into water had disappeared, the fish were placed into the aquarium. The total number of catching movements over each field per unit of time was recorded. The experiments were carried out with blinded and healthy fish. This method was also used for studies on food preference in ruffs.

For studies on food preference in eels, Hartmann used linen cloth coated with food homogenates immersed vertically on rods in the aquarium.

Studies on perception of various taste substances. If the natural reaction to the substance studied does not appear, taste sensitivity to this substance can be studied by means of conditioned reflexes. The method of conditioned reflexes allows one to establish what chemical substances are taken by the fish, and the relative threshold concentration of various substances perceived by the fish. The problem as to whether fish can discriminate between some chemical substances can also be studied by means of the method of conditioned reflexes.

It is difficult to administer the various chemical substances in strictly known doses. Strieck (1924) studied the capacity of minnows to discriminate sweet taste from bitter, sour, and salty tastes. The following method was used. Fresh sliced beef meat was placed in small crucibles into which 10 ml of one of the following solutions were poured, 40% glucose, 2% acetic acid, 2 - 10% quinine or 40% NaCl solution. Meat was soaked in these solutions for 0.5 - 1 hour. Portions of the liquids were filtered off and transferred into other crucibles with cotton. Fish were given pieces of meat soaked in the above solutions, and the inedible cotton was soaked in these solutions, or in meat juice. Meat juice always comprised a stimulus upon feeding and Strieck endeavored to eliminate this factor as a signal. For this purpose the author mixed meat juice with negative stimuli. Thus, the action of a stimulus such as glucose + meat juice was reinforced by meat which the fish found in the crucible, the action of differential stimuli such as meat juice + acetic acid or quinine were not reinforced. Meat was suspended in the aquarium on glass holders for one minute. The substance tested was discharged from a pipet to the bottom of the aquarium. The various stimuli were squirted into the aquarium one after another at 5-minute intervals. The experiments were carried out on blinded fishes. Their catching reactions were recorded. Differentiation could be developed in the course of 3 - 4 weeks, but the best results were obtained after 6 - 8 weeks. If bitter taste served as a positive signal, the fish soon died from exhaustion (they refused to eat bitter meat).

For tentative determination of relative thresholds, the following method was used. Fish were trained to some taste stimulus and were placed in a vessel of 1000 ml volume and 20 cm diameter. In the center of the vessel

a tube open at both ends was suspended. The lower end of the tube stoppered with a 1.5 cm cotton plug was about 8 cm from the bottom of the aquarium. Inside the tube were placed 5 ml of meat juice and the desired concentration of the taste substance which served as signal. The solution gradually diffused to the bottom of the aquarium. The reaction of the fish was observed for one minute. The experiment was begun with low concentrations of the taste signal. With each experiment the concentration of the latter increased.

Although this method is of certain interest, it does not allow one to establish the accurate value of concentration thresholds. Krinner (1935) used another method. Fish (minnows) were trained to transfers from an aquarium filled with pure water into aquariums filled with various solutions. This method enabled one to assess the threshold concentrations of various substances with a greater accuracy than that possible in the preceding method.

#### METHODS OF STUDY ON THE SENSE OF SMELL IN FISH

The sense of smell may be studied in fish with the aid of unconditioned and conditioned reflexes, and with the aid of electrophysiological studies on receptors.

Studies on the role of the sense of smell in the search for food. To verify the role of smell in fish in obtaining food, the method of hidden baits is used, alternatively the general excitation of fish on saturating the water with the smell of food is noted.

There are several methods of giving hidden baits. In his studies on the reaction of sea burbot to smell, Bateson (1890) had meat in opened bottles. The most widely used methods consist of the careful placing of food in an aquarium which contains blinded fish or fish with poor eyesight (Sheldon, 1911; Wunder, 1927; Andriyashev, 1944a, 1955 and others). Another method consists in suspending food wrapped in gauze envelopes in the aquariums (Parker, 1910, 1911; Ripping, 1926, 1927; Sato, 1937, 1937a, 1937b, 1938; Aronov, 1960, 1961).

In these experiments, the ability of fish to react to the smell of food which diffuses through water or is carried by a weak water current, as well as the capacity of fish to find food by means of smell, are recorded. The bait should be placed as far from the fish as possible, in order to avoid a chance effect of meat juice of high local concentration which is capable of bringing about excitation of taste receptors. It should be remembered that meat and meat juice from mollusks may undergo changes in sea water which result in a diminished activity of these stimuli on the taste and smell receptors (Bateson, 1890; Herrick, 1903; Aronov, 1959a). These changes occur in the course of 10 - 15 minutes.

In experiments with smell stimuli, the state of the water in the aquarium must be considered. Herrick showed that bullheads found bait by means of smell with difficulty in stagnant water, but discriminated between different odors with ease whilst swimming against the current.

Envelopes from gauze are prepared in the following way: two pieces of gauze, of one or two layers, are filled with identical amounts of washed, coarse sand or small pebbles, while one of them also contains a certain

amount of sliced meat. Both envelopes are tied and suspended in an aquarium in which a group of fish is present, the fish should swim approximately at the same water level, at some distance from each other. In outer appearance the envelopes should be indistinguishable. In the course of the experiments, the position of the envelopes should be changed. To estimate the ability of fish to find hidden food, the number of catching movements (catching of empty and food-containing envelopes) is recorded. Experiments should be carried out for 10-15 minutes or less, depending on the intensity of the reaction. The duration of the experiment should be limited to avoid formation of conditioned reflexes to the location of food-containing envelopes. Sato (1937, 1937a), however, carried out similar experiments for one hour.

In the experiments with envelopes the possibility of distinguishing between them with the aid of well-developed barbels should be considered (Sato, 1937a). Control experiments must be made on fish in which the sense of smell or vision is eliminated, or in which the barbels are cut off.

Studies on general alimentary excitation in response to smell is conveniently carried out on fish which search for food actively (sea burbot, and others). D.S. Pavlov described a method (personal communication) for studies on reaction of these fish to smell. To an aquarium one adds water which has a food odor, and the time of active swimming of the fish is recorded. In preliminary experiments, the duration of swimming of the fish in an aquarium filled with odorless water is determined. The odor of various edible products serving as food for the fish in question may be used (in this case, living atherinids). These organisms were kept in water for some time. Odor concentration may vary, depending on conditions: the volume of water in which the odor-producing organism is placed, its size, the time of keeping the fish in water, its activity, etc. This is a relative, quantitative method for assaying olfactory sensitivity in various fish species.

Studies on the role of the sense of smell in defense reactions of fish. Olfactory stimuli may also cause defense reactions. Frisch (1941) described specific substances present in the skin of minnows which cause an "escape reaction" in these fish. Frisch developed a simple method for determining the concentration of substances causing an escape reaction. A piece of skin weighing 0.2g from a killed minnow is cut into equal pieces with scissors and placed in a vessel with 200 ml of tap water at room temperature. The solution is stirred every 5 minutes and filtered after 30 minutes. Minnows in the aquarium should be trained to take food from a tube, near which they collect. During the experiment, 100 me of the above solution are poured into the aquarium. One minute later food is placed into the aquarium and the behavior of the fish is recorded. Frisch pointed out that the defense reaction in minnows (escape from food and hiding) was noted when the initial solution was diluted to 1:200 and sometimes to 1:500.

When the sense of smell is eliminated, minnows display no escape reaction or exhibit a short-lived state of alarm.

Studies on selective reactions to various smells. Wisby and Hasler (1952) constructed an apparatus for studies on the reaction of salmon to various organic odors. The apparatus consists of a central basin into which four canals lead. Water flows in the upper segments of each canal, cascades down and passes through 8 waterfalls. It then enters the central reservoir and overflows from it. Canal inlets from the side of the central compartment are covered by a door which can easily be lifted by

pulling a wire. Salmon fry are placed in the central compartment, one of the canals is filled with the odorous solution and the doors are opened. Fish distribution in control and experimental aquariums is then recorded.

Methods of conditioned reflexes used in studies on the sense of smell in fish. The method of conditioned reflexes has been widely used in studies on various problems connected with olfactory sensation in fish (Strieck, 1924, Wrede, 1932, Sanders, 1940, Göz, 1941, Neurath, 1949, Walker and Hasler, 1949, and others).

We shall describe here the apparatuses used by Göz (1941) and Hasler et al., (1954) for developing conditioned stimuli to odor. The apparatus of Göz is very simple, and can easily be constructed in any laboratory. Göz studied the ability of minnows to discriminate the smells of various fish and even of individuals of one and the same species. The device consists of two aquariums placed one above the other (Fig. 1). The experimental fish (blind minnow) is placed in the lower aquarium, while the upper aquarium contains the source of smell (crucian or brown bullhead). The lower aquarium is supplied with a constant stream of pure water which can be directed during the experiments into the upper aquarium by turning a bent tube upward and opening a stopcock (shown in the middle part of Fig. 1). This ensures the flow of water saturated with the given smell into the aquarium containing the minnows, without having to stop the flow of water.

The rate of flow should remain unchanged. When the experimental fish swims beside the stream of water flowing into the aquarium, food is offered to it (food is placed in the aquarium on a thin rod). When a differentiating odor is applied, the minnow is lightly tapped with a glass rod. This experiment is repeated several times a day.

The apparatus constructed by Hasler (1954) is more complicated. It consists of a 28 liter aquarium with an air-siphon circulation system (Fig. 2). Water flows out of the aquarium through siphons placed at both sides of the aquarium and is forced upward by air pressure along two vertical tubes into funnels fixed along two opposite corners of the aquarium. Each funnel is connected with a tube which runs downward and passes across the bottom of the vessel. Openings in these tubes direct the water flow along the bottom of the aquarium. The streams flowing one against another deviate upward and backward when they meet in the center of the aquarium. These currents form two compartments, two convection chambers, each of which occupies one half of the aquarium.

Odorous solutions are introduced into the aquarium from two separating funnels connected to the siphons by means of tubes. During intervals between experiments, water circulating within the siphon system is passed through activated charcoal to remove its odor. This is necessary for the elimination of olfactory adaptation in fish. Activated charcoal is packed in two wide glass columns, 4-5 cm in diameter and 50 cm in length. It has been experimentally shown that trained fish do not respond to odors after the odorous solutions had passed through such filters.

Three carbon electrodes are placed on each side of the aquarium. The distance between them should be 5-7 cm. The space between the electrodes is called the "final zone" which serves for feeding fish and stimulation by electric current, depending on the significance of the odor. Food is given in a feeding rack covered with perforated celluloid plate. The electric current is of 11.5 milliamperes and 1.5 volts.

Experiments were carried out on fish blinded by injecting phemerol into the posterior eye chamber. To avoid rocking, the aquarium is placed on porous rubber.

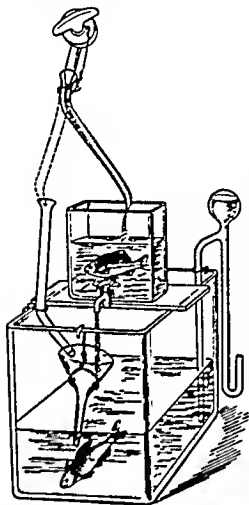


FIGURE 1 Apparatus for developing conditioned reflexes to odor in various fish (after C&Z, 1941)

Electrophysiological studies on receptors for the sense of smell in fish. Adrian and Ludwig (1938) used the electrophysiological method in their studies on the response of olfactory receptors of brown bullhead, carp and tench. Experiments were carried out on surviving organisms devoid of blood circulation. The fish examined was decapitated and its head was pinned to a rack. The fore-brain was exposed and lifted on a small ring of chlorinated silver wire, the olfactory trunk remained suspended in the air in the process. A second silver electrode was inserted into the olfactory bulb which was split by thin needles. The second electrode could also be inserted into other parts of the head. The heart was then removed. Such preparations were obtained in a matter of 5 minutes, and then the fluctuations of electric potentials were recorded for 30 or 60 minutes. Under such conditions, irrigation of the gills with water in some preparations in which the blood circulation was preserved did not increase the activity of the olfactory system. Registration of impulses was done in the way described earlier.

Olfactory stimuli were applied in two ways. In the simpler case the stimulating solution was dropped or squirted into the olfactory sack and was washed out with the aid of a stream of water. For the sake of convenience the cover of the sack could be removed (but the organ was better pre-

served if the integrity of the sack was intact). Injection of liquid ensured a rapid increase in the concentration of the stimulus near the receptors, and allowed for application of a strong stimulus. The drawback to this method lies in the fact that the olfactory organ is acted upon mechanically, and in that the recording is unsatisfactory.

The second method entails a continuous supply of water into the nostrils of the fish through a glass cannula. By closing and opening the appropriate stopcocks, water could be replaced by odorous liquid. The cannula should be fixed on a heavy tripod (to prevent dislocation of the cannula during manipulations with stopcocks, the correct location of cannula was secured by means of screws). The method of "continuous stream" gives a better recording than the preceding method.

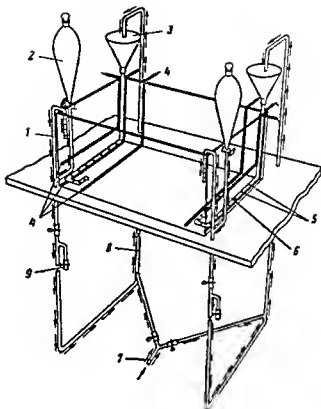


FIGURE 2 Apparatus for studies on the ability of fish to differentiate various odors (after Hasler, 1954)

1-siphon, 2 separating funnel for odorous solutions, 3 funnel for water desaturation, 4-electrodes, 5-directing tube, 6 feeding rack, 7-air hose, 8-air pressure tube, 9-stopcock

Olfactory organs responded both to chemical and mechanical stimulation. An essential drawback of the above electrophysiological method is that Adrian and Ludwig could record responses to high concentrations of odorous substances only, while under natural conditions the olfactory organ is acted upon by negligible concentrations of odor.

#### METHODS OF ELIMINATION OF RECEPTORS

In the course of experiments a necessity of eliminating one or two chemical receptors may arise. To avoid side-effects, numerous workers blind the experimental fish.

Elimination of vision. Fish may be blinded by radical removal of both eye-balls, galvanocauterization of the cornea, removal of the lens and injection of phemmerol into the posterior eye chamber. In the latter case



about 0.03 ml of undiluted phemerol is used. This procedure gave good results with muraena eel, for example. (Bardaeh, Winn and Menzel, 1959). Sato (1937a) blinded fish by sewing blinders from thin kid-skin to the skin of the head in three places.

All operations are conveniently performed on fish wrapped in wet gauze and held in one hand while all manipulations are done with the other hand.

**Anesthesia.** The fish may be anesthetized before the operation. If the duration of the operation is less than 5-7 minutes, anesthesia is not required.

German workers prefer urethane anesthesia - 0.5% solution of ethyl urethane (Strieck, 1924, and others). In marine fish ether anesthesia can be used (Sato, 1937b, 1938, Andriyashev, 1944, 1944a, 1944b, Aronov, 1959a). Sato used 5-10% ether emulsion, other authors found that 0.5-1.0% ether emulsion was satisfactory.

For anesthesia the fish is placed in a closed crystallizer with a known volume of sea water. Ether emulsion is prepared in a glass-stoppered bottle by mixing ether sulfate with water (ether concentration should be such as to give the required concentration after adding it to the vessel with the experimental fish).

Immediately after adding ether, the fish becomes excited but soon ceases to move. Usually 1.5-2 minutes are sufficient for anesthesia. Cessation of respiratory movements, lying on the side or back, and loss of reaction to touch, show that the depth of anesthesia is adequate. In operations of long duration, water flowing to the mouth of the fish to sustain respiration may contain a low concentration of the anesthetic drug used. In such a case the fish should be fixed on the operation table. Various methods are available for this purpose.

Anesthesia of short duration has no deleterious after-effects, cases were noted in which gray mullets and whiting commenced to take food 1-3 hours after an operation performed under anesthesia (Aronov, 1959a, 1960). Usually the fish begin to take food 2-3 days after the operation, and in some instances only one or two weeks after the operation and later.

If the fish is to be operated on without anesthesia, it should be wrapped in wet gauze and its head should be fixed in a loop made of gauze. Only the surgical field remains uncovered.

**Taste receptors.** Usually only external receptors are eliminated. The methods available are not always adequate, although in numerous instances they may fulfil the conditions required. External receptors may be eliminated by the destruction of taste buds with acids (Andriyashev, 1944b, Aronov, 1959a), removal of barbels (organs bearing taste buds) (Parker, 1910, Sato, 1937, 1937a, 1937b, 1938, Andriyashev, 1944b and others), by cutting of nerves which innervate the taste receptors (Olmsted, 1920; Dykgraaf, 1934, Aronov, 1959 and others).

For the destruction of taste buds by means of acid the fish are taken out of the water and their lips or barbels are treated with 5-7% hydrochloric acid solution. This procedure destroys the taste receptors for several days, the completeness of destruction of the taste buds is difficult to check.

Although removal of barbels eliminates the function of this highly specialized organ, it does not eliminate external taste sensation in the mouth region, since taste receptors may also be localized in the epidermis of the lips and snout.

Sectioning of certain nerve branches is the most reliable method of elimination of taste reception of the appropriate segment of skin. Sensitivity of these segments to strong stimuli, however, may be preserved (to acids, for example), owing to stimulation of the endings of nerves of general skin sensation (Parker, 1912).

When natural stimuli such as food are applied, the most reliable method for elimination of taste receptors is that based on denervation.

Sectioning of nerve fibers in the head region is a difficult procedure. In each case the anatomical features of the nerves must be known. Olmsted (1920) obtained a preparation of brown bullhead head by treating it with 30% solution of nitric acid. In such a preparation the muscles are easily removed while the nerves remain intact. Vital staining of the nerves with methylene blue may also be used.

It is much simpler to section the recurrent branches of the facial or accessory nerves which innervate the taste buds of the fins and body of cods, carp and other fish. In *Gaidropsorus* (sea burbot) these nerves are easily exposed by peeling the skin of the upper part of the head (Aronov, 1959). Nerve fibers are cut by scissors at their point of exit from the cranium. Such an operation can be performed according to a special procedure by a short incision through the skin. Usually the nerves are sectioned on one side of the fish, while the other side serves as a control. At the end of the experiments the results of sectioning of the nerves are confirmed under a binocular microscope.

It should be remembered that the sectioned nerves may regenerate after some time. Olmsted (1920) showed that the taste buds in brown bullheads located at the base of the barbels and which disappear after sectioning the nerve, reappeared toward the eighteenth day, and the function of the barbel was restored towards the 40th day after sectioning the nerve.

Receptors for sense of smell: the nostrils may be covered by various materials (Sheldon, 1909; Wunder, 1927; Andriyashev, 1944, 1944a and others; Bardach et al, 1959; Aronov, 1959a and others); the anterior nostrils may be sewn by a ligature from thin silk thread (Parker, 1911); the olfactory rosette may be cauterized (Andriyashev, 1944a; 1944b, Walker and Hasler, 1946; Aronov, 1959a); the olfactory nerves may be cut (Herrick, 1903; Parker, 1910, 1911; Sato, 1937a; 1937b; 1938, Andriyashev, 1944a). The olfactory bulbs may be removed (Gözl, 1941) and the fore-brain may be extirpated (Strieck, 1924).

Sheldon (1909) covered the nostrils of dogfish with cotton or with cotton and vaselin; Wunder (1927) introduced into nostrils of pikes thin plasticine rods which exactly fitted the opening of the nostrils. At the distal end the plasticine rods were widened and fixed with a ring. The plasticine rods can easily fall out of the nostrils. Trout had their nostrils closed by parts of pins, which could easily be removed (Wunder). It is not recommended, however, to use metal plugs in marine fish because of metal corrosion in sea water. A. P. Andriyashev (1944a) recommended the use of aluminium wires as used by him in his experiments on sea burbot.

The results of galvanocauterization should be checked under the binocular microscope.

The olfactory nerve may be cut from a small incision through the roof of the cranium. The exact localization of the incision is determined by the anatomical features of the fish in question. Parker (1911) cut the olfactory

nerve of *Fundulus heteroclitus* between the eyes of the fish. Best results were obtained when the operation was carried out without anesthesia. Sato (1937a, 1937b, 1939) eliminated the sense of smell in Japanese red mullet by an incision behind the olfactory hollow, and in sea sheathfish by an incision behind the nasal barbel. These operations were carried out under ether anesthesia.

The sense of smell may also be eliminated by removal of the olfactory centers, i.e. by extirpation of the fore-brain (Strieck, 1924). A.V. Baru (1955) and G.A. Malyukina (1954) used a rapid and simple method for extirpation of the fore-brain which has numerous advantages over the painstaking method of Strieck, and which can be used successfully in experiments with both fresh-water and marine fish.

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## METHODS OF STUDY ON THE LATERAL LINE ORGAN OF FISH

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The lateral line organ of fish is a specific analyzer of animals adapted to the aquatic way of life. With the aid of the lateral line, fish perceive water vibration of low frequency (Parker, 1905, Rode, 1927, Disler, 1960, and others) and differentiate them according to frequency and direction (Malyukina, 1955, 1958). Perception of weak streams of water is also carried out by the lateral line organ (Hofer, 1908, Dykgraaf, 1934) as well as a distant perception of stationary hard bodies (Dijkgraaf, 1947, Disler, 1960). In many predatory fish the lateral line organ plays a leading role in the search for food (moving prey) (Wunder, 1927, Andriyashov, 1944, 1944a).

In the majority of bony fish the lateral line organ is represented by a system of subdermal canals on the head and by two symmetrical canals running along the body of the fish, in the cavity of which the sense organs are located. In addition, the body and head of the fish are covered with superficial sense organs. The structure of the lateral line in crucians (*Carassius*) is shown in Figure 1. The lateral line organ in the head of fish is innervated by branches of the trigeminal, facial and glossopharyngeal nerves, and on the body by a branch of the vagus and lateral nerves.

One of the methods of study on the function of the lateral line organ is that based on observations on reactions of fish to various stimuli which are very similar to water currents, waves, etc.

Before the experiments the fish are blinded. To assess the perception of various stimuli, characteristic reactions of the animal are used: change in position and fins of body under the influence of vibrations of low frequency (Parker, 1905), turning of body when the fish is squirted with a thin stream of water (Hofer, 1908, Dykgraaf, 1934), catching movements of fish in response to water vibrations created by small bodies (Wunder, 1927, Andriyashov, 1944, 1944a, Disler, 1960), movements of the fish away from the stimulus, for which a large body in the water is used (Dijkgraaf, 1947, Disler, 1960), etc.

The method of observation successfully employed in studies on the role of the lateral line organ in alimentary and defense reactions in fish has enabled us to assay the biological significance of the stimuli perceived. This method is impractical in the absence of clear-cut reactions to stimuli. In such instances, as well as for more comprehensive studies of the sensitivity of the lateral line organ, the method of conditioned reflexes ("method

of training" according to the terminology of foreign authors) is used. Without dwelling on the principle of this method which has already been described in this monograph, we shall point out certain features of conditioned stimuli and the methods of their application.

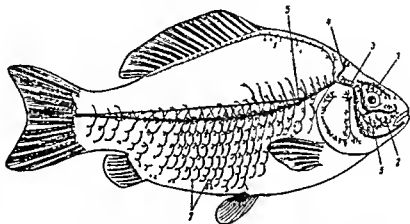


FIGURE 1. Structure of lateral line in *Carassius carassius* (after Disler, 1960)

1—supraorbital canal, 2—infraorbital canal, 3—temporal canal, 4—supratergital canal, 5—preoperculo-mandibular canal, 6—lateral canal, 7—free-living organs

Weak water currents and water vibrations of varying intensity and frequency serve as conditioned stimuli. Vibrations may be generated with the aid of a simple device. A noiseless electric motor is fixed on a rubber gas-ket. Rotation of the motor is conveyed to a horizontal shaft with a cam at its tip. Rotation of the latter causes vibrations of a frame with a plexiglass rod. The amplitude of oscillations of the lower end of the rod in our experiments (Malyukina, 1955, 1958) never exceeded 2 mm. The speed of rotation of the shaft can be regulated with the aid of a rheostat. Before the experiment, the plexiglass rod should be immersed in the aquarium and screened from the fish. A rigid frame with a thin opaque rubber stretched over it can serve as a screen.

Kleerecoper and Chanson (1954) suggested that the apparatus shown in Figure 2 should be used for obtaining low-frequency vibrations.

A metal box (1) is covered with rubber membrane (2) connected with a tube and rubber cup (3) with a thin plastic membrane. Cup (3) is placed in the aquarium and serves as the source of vibrations. Tubes (4-7) lead to similar cups in other aquariums. The entire system is filled with water. To eliminate sounds caused by compression and expansion of air, air bubbles are carefully removed from the system. Membrane (2) is connected with rod (8) of a sewing machine from which the needle holder has been removed. The flywheel is replaced by a pulley which is set in motion by an electric motor. The speed of rotation of the pulley is regulated with the aid of a rheostat.

Conditioned reflex from lateral line organs is developed by the conventional methods. Experiments are carried out in aquariums in which the experimental fish usually lives. At the moment of application of the conditioned



stimulus the distance between the fish and the vibrator should be always the same. If the vibrator produces even the slightest noise or additional vibrations one must check whether the conditioned reflex is not directed to these stimuli. For this purpose the vibrator is switched on but no vibrations are produced and the reaction of the fish is noted. If the fish does not respond to this stimulus the experiments can be continued.

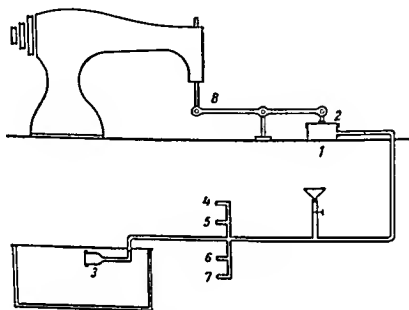


FIGURE 2 Device for obtaining low frequency vibrations

To verify whether the conditioned reflex is really from the lateral line organ and not from other sense organs control experiments are required. If the vibrations used have the frequency of sound (or close to it) one must check that the organ of hearing does not participate in formation of the conditioned reflex. For this purpose in experiments of long duration the auditory segment of the labyrinth is removed (for method of removal of the labyrinth) and the fish is tested for the appearance of the reflex (see p. 280).

To check if the vibrations applied are not perceived by skin receptors the lateral line organ should be eliminated. This is best done by denervation (Frisch and Stetter 1932). The fish is placed in a 0.5% solution of urethane. The anesthetized fish is taken out, wrapped in wet gauze and fixed on the operating table. A glass tube is placed in its mouth. Through this tube pure water or 0.25% aqueous solution of urethane is perfused depending on the depth of anesthesia. Ether anesthesia may also be used (the fish is placed into a 2% emulsion of ether sulfate in water for several minutes).

To expose the lateral nerve near its point of exit from the skull the gill cover should be opened and pulled aside. Muscles lying over the fourth gill arch (somewhat to the right of the latter) are separated by hooks exposing the root of the lateral nerve. This nerve should be sectioned so that its dorsal branch which forms near the entry of the lateral nerve into the skull should be below the cut. As a result of this operation all intracanalicular and free lying organs of one side of the fish are eliminated. To avoid regeneration as great a segment of the nerve as possible is removed.

Nerves which innervate the canal and free lying sense organs of the head are sectioned in several steps. The gill cover is pulled aside as far as possible and the opercular branch of the facial nerve on the inner side of the gill cover is cut near its entry into the skull.

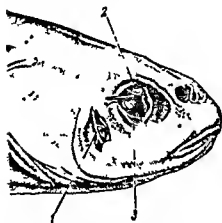


FIGURE 3 Arrangement of branches of facial and trigeminal nerves which innervate the lateral line organs of the head (Frisch and Stetter 1932)

1—opercular branch of facial nerve 2—ophthalmic branch of trigeminal nerve 3—infraorbital trunk (maxillary and mandibular branches of trigeminal nerve)

Fibers of the nerve of the lateral line comprising the trigeminal nerve are located inside the eye socket in trunks of the ophthalmic and infraorbital nerves (Figure 3). To cut these branches the eye bulbs do not have to be removed as proposed by Frisch and Stetter. The intraorbital nerve branches may be cut if the organ of vision is fully preserved. For this purpose the conjunctiva is cut around the eye; its lower edge is held by anatomical forceps and the eye bulb is pulled upwards. The thin muscle layer of the lower part of the eye socket is separated by small hooks and the infraorbital trunk is hooked and cut. The upper edge of the conjunctiva is held by forceps and pulled downwards. The ophthalmic branch is then found and cut. An identical operation is then carried out on the second eye. Thus operation leaves the sense organs of the supra-

temporal canals and small groups of free lying organs located in the vicinity of these canals intact. Sectioning the nerve branches which innervate these organs is extremely difficult; they are therefore eliminated by cauterization.

One day after the operation the fish are indistinguishable in behavior from healthy ones. After the fish have begun to take food they may be used for testing previously developed reflexes.

At the end of the experiment the fish must be dissected to check whether the organ has been completely denervated.

Several workers have studied the function of the lateral line organs by means of the electrophysiological method.

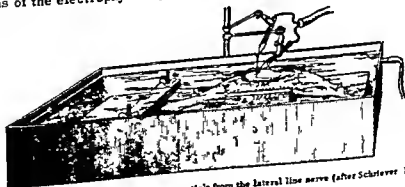


FIGURE 4 Apparatus for studies on biopotentials from the lateral line nerve (after Schriever 1935)

Hoagland (1933, 1933a) sectioned the central nervous system at the level of the medulla oblongata before the experiments. Skin and muscles above the lateral line canal of the body were dissected, and a 1 cm segment of the lateral nerve was exposed, ligated, and the proximal nerve end cut. The fish was immersed in water so that the lateral line organ of the body was covered with water. The nerve was moistened by Ringer's solution and hooked onto chlorine-silver electrodes. The electrodes were connected to the oscillograph through amplifiers. A loudspeaker was included in the circuit through an additional amplifier.

Schriever (1935) placed the fish in a urethane solution (5g urethane per 1000 ml water) before the operation. The anesthetized fish was fixed at the bottom of the vessel as shown in Figure 4. The lateral nerve was exposed and its proximal end hooked onto electrodes. After the operation, the urethane solution was replaced by fresh water in which the fish became alert. The water should be replenished several times in the course of the experiment. Schriever pointed out that respiration and circulation of the operated fish should not deviate from the norm, disturbances in respiration and circulation result in a decrease in the sensitivity of the lateral line organ. Tapering glass tubes filled with Ringer's solution for fish\* served as electrodes. The thin end of the tubes was plugged by cotton wool. From the other end a chlorine-silver wire was inserted. Biopotentials were recorded in the same way as in the experiments of Hoagland.

In these experiments the following stimuli were used, light touch of the skin above the lateral line canal with a bird feather tip of Frey hairs, stream of water directed into the region of the canal; water vibrations of low frequency, vibration of vessel walls, temperature and chemical stimuli.

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\* Schriever suggested the following solution: NaCl, 2g, NaCl, 0.2g, CaCl<sub>2</sub>, 0.2g, water, 1000ml.

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## METHODS OF STUDY OF BIOELECTRIC BRAIN ACTIVITY

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For investigations of the laws governing the activity of the central nervous system numerous physiological laboratories use the electrophysiological method

Recording of biopotentials from fish brain is connected with a number of technological difficulties. Following is a simple method for recording bioelectric activity of various segments of the fish brain in short- and long-term experiments.

1 Recording bioelectric potentials of the fish brain in short-term (acute) experiments. Almost all experiments on the bioelectric activity of the fish brain were carried out under conditions of acute experiments or even on brain preparations (Adrian and Ludwig, 1938, Buser, 1949, Woldring, 1950, and others).

The results obtained with brain preparations isolated from the skull and devoid of blood circulation (in preterminal stages of life) should be interpreted with caution. This has been pointed out by those scientists who applied this method. For this reason we shall not dwell on the methods of obtaining the brain preparations and recording their biopotentials.

We shall describe in greater detail the method of recording of fish brain potentials in acute experiments

The fish should be fixed in devices of two types. Woldring and Dirken (1951) and other authors fixed the fish in a special vertical plate constructed as a splint which fitted the fish body and was provided with openings for gills. The fish was held by special collars which encircled the fish body in several places. Water enriched in oxygen was perfused into the mouth of the fish through a special cannula. The fish fixed in this way was placed in the aquarium with its head above the water surface. Urethane, nembutal or other drugs may be used for anesthesia. Narcosis, however, often resulted in cessation of respiration and death of the fish.

In our experiments we never used anesthesia. Instead, the fish was fixed in a special device provided with a special head-holder (Figure 1). This device prevented any dislocations of the electrodes during respiration. The device for fixation of the fish consists of base (1) and arches (2) of thick brass wire which may easily be twisted into any form. Each arch can be moved around its axis completely freely in relation to other arches. This allows one to fix fish of varying size. When very large or very small fish

are to be fixed, the arches may be removed and replaced by shorter or longer ones. For this purpose each arch may be easily screwed off the hinge (3). In addition, the base of the fixing device may hold any number of hinges which enables one to fix fish of any length. To do this it is sufficient to add the desired amount of hinges with arches. The hinges are tightly connected with each other by a nut (5).

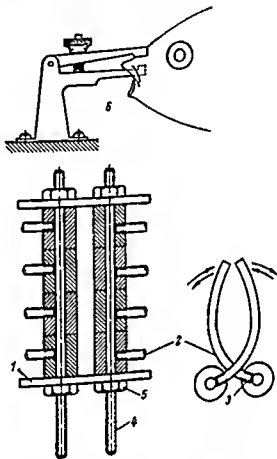


FIGURE 1 Clamp for head and fixation device for body of fish (explanation in text)

This device has numerous advantages over other types of fixing devices in that the body of the fish is not damaged by being fixed by rubber covered arches (Figure 2). The fish should be fixed so that its head together with the gill covers should not be encircled by arches (Figure 2).

A special clamp serves as head holder (Figure 1, 6) this is placed in the mouth of the fish, the upper part of the head is held tightly, while the mouth parts can move freely. Normal respiration is thus ensured. Interchangeable head holders may be used for different fish species. In the case of fish of one and the same species but of varying size, the holder can be moved vertically by means of screws. The advantage of this method of

fixation lies in that the head is rigidly fixed while allowing free access to all parts of the brain

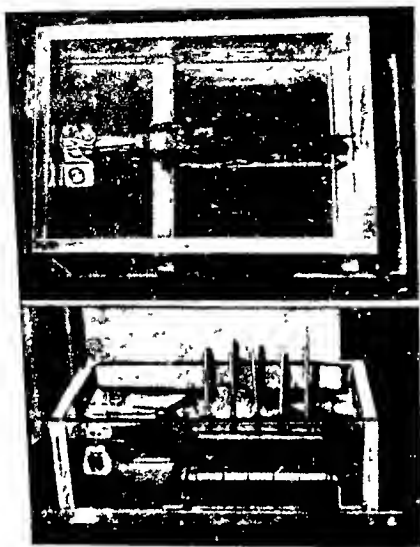


FIGURE 2 General view of fish fixed in the fixing device top-view from above, bottom--lateral view

The fish together with the fixing device and head-holder is immersed in a special plexiglass aquarium which is filled with water. The skull of the fish should remain above the surface of the water. The skull is then carefully dissected, the fatty layer is carefully removed and the brain exposed.

For recording respiration in fish we have used 1) a piezocrystal, 2) thermistor, and 3) solenoid with coil. 1) The gill cover is connected with a rubber-covered Marey capsule by means of a thin silk thread. The piezocrystal is fixed on the inner side of the Marey capsule (Figure 3). All movements of the gill covers are transmitted through the piezocrystal and recorded on a loop oscillograph.

2) The second method is preferable, since it gives more accurate recording of all movements of the gill covers. This method is based on the

fact that the thermistor is fixed at the exit tube of the Marey capsule (Figure 3). Respiratory movements were recorded by the method of A. A. Volokhov, V. I. Kobysh and E. G. Novikova (1956) based on movement of air in the capsule.

3) The third method of recording involves the connection of the gill cover with a miniature solenoid through a silk thread and a pulley. The solenoid can move along the coil (Figure 3). The terminals of the coil windings run to the amplifier. Solenoid and coil may be replaced by a small amperimeter. The gill cover is connected with the pointer of the amperimeter by means of a silk thread. The entry points of the terminals of the amperimeter are connected with the input terminals of the amplifier.

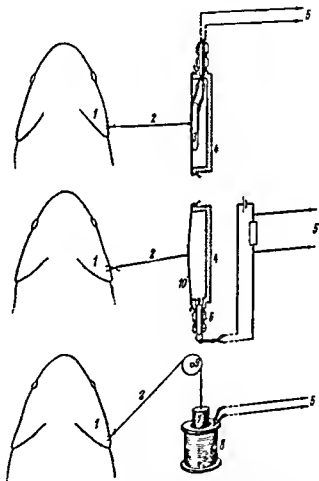


FIGURE 3. Various methods for recording fish respiration  
1—gill cover; 2—thin thread; 3—piezocrystal; 4—Marey crystal;  
5—amplifier input; 6—thermistor; 7—solenoid; 8—coil windings;  
9—pulley; 10—thin rubber.



In our experiments with fish the aquarium was fixed on a MM-1 micro manipulator (Figure 4) or on a specially designed stereotaxic device for lower vertebrates (fish, amphibia, reptiles, birds and rodents). For obtaining biopotentials from the brain surface it is convenient to use micro-manipulators, since two pairs of electrodes can be fixed in its tripod. Each of the electrodes can be moved separately (Figure, 4, 1 and 2). Brush electrode made of a cotton thread, inserted into a thin spiral from platinum or chlorine-treated silver wire, served as surface electrodes.

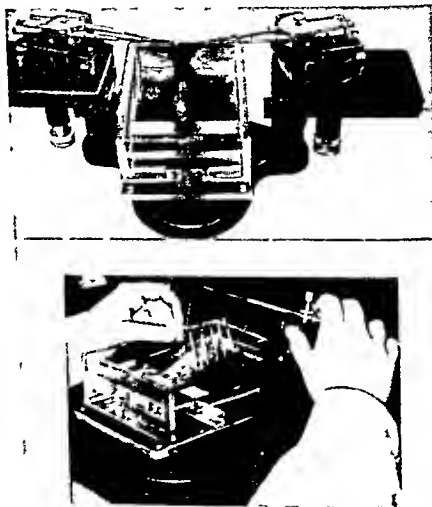


FIGURE 4 Position of fish in short-term (acute) experiment (aquarium fixed between plates of micromanipulator)

In experiments with fish it is extremely difficult to use the monopolar method of recording potentials. In this case the electrograms from all brain parts are complicated by vast artifact waves and discharges connected with respiratory movements of the gill covers. Numerous authors (Enger, 1957 and others) have noticed similar artifacts in electrograms of fish and certain amphibia.

The electrodes are moistened in physiological saline and placed on the brain. For obtaining of potentials from the deeper part of the brain with

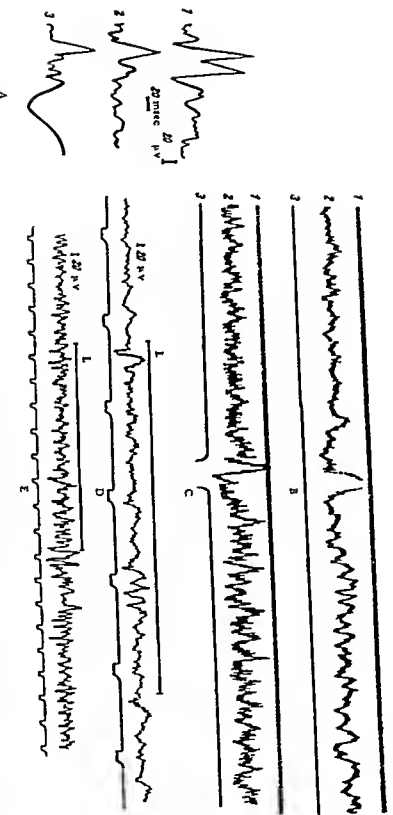


FIGURE 6. Bioelectric reaction in various parts of the brain in fish, recorded in short-term (acute) experiment (A, B, C) and in long-term (chronic) experiment (D, E). A—electric reaction in electrogram of tectum opicum (1), cerebellum (2) and anterior brain (3) of gold fish to flash of light of 5 joules intensity (light was applied to the eye on the opposite side of the brain part studied). B—reaction of enhancement of rhythm in electrogram of anterior brain of gold fish to continuous white light and C—to near light, 1—time, 50/second, 2—encephalogram of anterior brain, 3—application of stimulus (light). D—changes in electrograms of cerebellum and E—of tectum opicum of midbrain of mirror carp (upon application of continuous white light, chronic experiment, time—1 second.

11 or 120 the electrode may be inserted (by means of a conventional micro meter) into the brain of the experimental animals with an accuracy of 0.5  $\mu$ . Such micromanipulators may be used in experiments with different animals. The method for preparing immersed electrodes has been described earlier (Gusel'nikov, 1957) and consists in dissolving nickel-chromium wire electrolytically. Oscillograms of fish recorded in short term (acute) experiment are illustrated in Figure 6.

2. Recording of bioelectric potentials in the fish brain in long term (chronic) experiments. We (in 1956) and subsequently Enger (1957), have succeeded in obtaining electroencephalograms from some parts of the brain of fish in long term (chronic) experiments with the aid of inserted electrodes. For inserting electrodes the fish is fixed in a device described above. The skin of the head is removed, the skull is defatted by ether and alcohol and carefully dried. Holes are then drilled with a watchmaker's drill in the appropriate places of the skull (for each species the calculation must be done previously). Through these holes the electrodes are inserted until they touch the brain. The electrodes are sealed with phosphate cement (used in dentistry) and a mixture of wax and vaseline.

Deflecting electrodes are of two types, depending on the aim of the studies. If the experimental fish can be fixed in the device by the method described above, the electrodes may be inserted so that only 1-1.5 cm protrudes above the cement. In such cases we have used the usual platinum or chlorine-silver wire with bulb-shaped thickening on the end and covered with lacquer along their entire length. The fish is so fixed that the electrodes protrude above the surface of water. The electrodes are carefully dried and connected with the entry of the amplifier by means of clamps. Such electrodes are held in position for a long time.

In experiments in which it is undesirable to fix the fish for each experiment, electrodes of somewhat different construction may be used. A light, lacquer-isolated wire is sealed to the upper ends of the electrodes (before the operation). The site of fusion is carefully covered with lacquer. Wires and electrodes are covered by a thin polyvinyl chloride tube 8-10 cm long. The upper end of the tube is tied to a float (Figure 7), while the lower end is fixed to the head with cement. The polyvinyl chloride tube can be waterproofed by a coating of wax or vaseline.

The fish is operated on and placed in a separate shallow aquarium. The thinner the polyvinyl tube, the more carefully the electrodes will be held in position for a long time.

For the experiment, the fish together with the aquarium is placed in the chamber. It must be added that rapid movements of the fish may cause artifacts in the EEG. An oscillogram of the carp brain obtained in long term (chronic) experiments is illustrated in Figure 6.



FIGURE 7 Fish with inserted electrodes  
1—float.

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## OPERATIONS ON BRAIN OF FISH

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Surgical removal of certain parts of the brain is used in various experiments on the examination of fish brain function of physiology of sense organs and of other problems of higher nervous activity. Usually the hemispheres of the fore-brain and the optic tectum and cerebellum are removed. By the virtue of its structure and localization the inter-brain can be destroyed only in part.

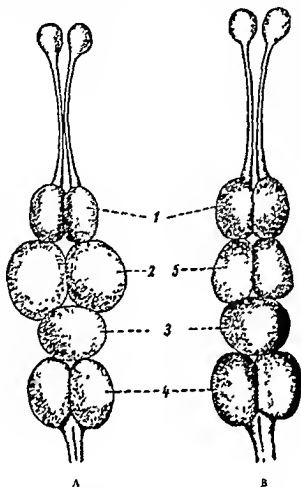
For operations on the central nervous system the fish should be given urethane or ether anesthesia, the best results, however, are obtained with natural gas, as proposed by A. I. Karamyan (1949).

When the fish is taken out of the water, it sinks into a narcotic state in a matter of few minutes. The fish is wrapped in wet gauze and held in the hand. The skin is removed from the site of incision, and a small hole is made into the skull with forceps. The hole should be located immediately above the segment of the brain which is to be removed. Fatty tissue which covers the brain should be pulled aside, if possible, or removed. If fatty tissue is not removed it is used after the operation to cover the operated segment. The fatty tissue should be removed by suction by means of a pipet (to minimize the possibility of damaging nervous tissue). The segment to be extirpated is then dried by applying small pieces of filter paper to the surface of the brain. No tampons should be used since they may cause local hemorrhages (especially in large fish).

In fish (see Figure) the fore-brain is clearly delineated and its removal presents no difficulties. An incision is made on the border between the fore-brain and inter-brain. The olfactory tracts which run to the nasal capsules are then cut, and the two hemispheres are pulled out with the aid of thin, anatomic forceps. Some authors sewed the skin-bone shield and poured gelatin or paraffin over the stitches at the end of the operation (Strick, 1925; Janzen, 1932; Hosch, 1936 and others).

After checking on the completeness of removal of the hemispheres, Karamyan (1949), Baru (1955) and Malyukina (1958) poured a mixture of wax and vaseline (2:1) over the hole in the skull. These authors later noticed that the state and behavior of fish in which the fore brain was removed, was unchanged when the hole in the skull remained uncovered. In time the hole filled with new bone tissue and 3-4 weeks after the operation the fish was

indistinguishable from the intact fish. The operation lasted several minutes, so that few postoperative complications can be expected.



Brain of *Carassius carassius*

1—corpora striata (hemispheres of fore-brain), 2—tecti optici, 3—corpus cerebelli, 4—lobi vagi, 5—valvula cerebelli.

After removal of the fore-brain the fish is placed in water and immediately begins to swim. It is, however, less mobile than normal fish, although it keeps the ability to respond to stimuli with rapid and energetic movements. After some time (from several hours to two days) the fish begins to take food actively.

**Tecti optici**—one of the most developed parts of the brain in bony fish. Divided by a longitudinal groove, they form a vault under which the valvula cerebelli is located. The structure of the tecti optici and their link with other brain parts as well as the closeness of the utriculus render their removal difficult.

Having dried the brain surface, the tecti optici are divided along the median line (this should be done carefully so as not to damage the valvula cerebelli). Then one tectum after another is lifted with the aid of thin anatomical forceps, without pulling up the brain, cut around by Wecker

scissors, and pulled out from the skull. This operation calls for maximum care not to damage the corpus and valvula cerebelli, and not to harm the utricle with the semicircular canals. Special attention must be directed to removal of the segments of the tecti optici which lie in the immediate vicinity to the inter-brain and corpus cerebelli where severe hemorrhages which are arrested with difficulty may be caused. If hemorrhages do occur, thrombi should be allowed to form, and are then removed with thin anatomical forceps. The surface of the brain is then rinsed with Ringer's solution. One must ascertain whether the tecti optici have been completely removed, the remaining segments should be removed with thin anatomical forceps.

Fish, the tecti optici of which are removed, are blind during the first days after the operation, they bump into the aquarium walls, after a short time, however, the fish begin to orientate well when in their movements.

Among the fish operated on, there are certain specimens in which motor activity increases markedly after the operation. Such fish swim around their axis, are exhausted and swim to the surface of the water and lie on their side. After some time this procedure is repeated. These phenomena described by several authors were considered by some to result from elimination of the functions of the midbrain. We are inclined to think that these phenomena result from certain central damage (pulling up of brain, thrombi, destruction or dislocation of the labyrinth, etc.). This assumption has been confirmed by the fact that similar disturbances have sometimes been observed after removal of the cerebellum or some of its parts.

When the operation is carried out carefully, the fish remained in good condition and no disturbances were noted.

The cerebellum of bony fish consists of two parts, the corpus cerebelli and the valvula cerebelli. The corpus cerebelli, the highest part of brain, is clearly delineated and usually overhangs the medulla oblongata.

Having dried the surface of the brain, a slightly posterior sloping incision is made on the border between the corpus cerebelli and the tecti optici. The corpus cerebelli is then pulled out from the skull.

The valvula cerebelli is located below the tecti optici in the optic ventriculum of the brain, and is thus accessible with difficulty. A. I. Karamyan (1949) developed a method for removal of the valvula cerebelli without damaging the adjacent brain parts. For extirpation of the valvula cerebelli, a thin hook or a twisted spear is put under one of the two halves of the tectum opticum, from the direction of the corpus cerebelli and directed clockwise (on the right side) or counter-clockwise (on the left side). Having hooked one lobe of the valvula cerebelli, the latter is pulled from beneath the tectum opticum, is separated from the corpus cerebelli with the aid of anatomical forceps, and is removed. The same procedure is then repeated with the second half of the valvula.

The completeness of removal is checked, and the hole in the skull is filled with a mixture of wax and vaseline.

Crucians and carps, the cerebellum of which has been removed, display disturbances of motor activity. The tonus of the muscles is markedly lowered. The fish lies on its side for a long time on the bottom of the aquarium or at the water surface. After some time it begins to swim, performing sharp swaying movements. It soon grows tired and again goes down to the bottom, where it rests. Apart from disturbances in motor function and coordination of movements, crucians and carps also display severe sensory disturbances which are manifest in considerable loss of tactile sensitivity.

and disturbances of vision and of the lateral line organ. On removal of the corpus cerebelli, the fish lies on its side at the bottom of the aquarium. After some time it begins to swim, swaying in its movements and bumping into the aquarium walls.

Fish from which the corpus cerebelli has been removed, display motor, sensory and trophic disturbances. Ulcers appear, scales fall out, and finally the fin rays fall out (Karamyan, 1949, Malyukina, 1954).

Removal of the valvula cerebelli causes less severe disturbances in motor activity than extirpation of the corpus cerebelli. Sensory functions are damaged more on removal of the valvula cerebelli than on removal of the corpus.

Several days after the operation the motor and sensory activity of the operated fish gradually return to normal.

If extirpation of the brain parts is combined with the development of conditioned reflexes, the fish which display recurrent disturbances should be excluded from the experiment.

At the end of the experiment the fish are killed and the results of the operation are checked histologically.

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# EFFECT OF LIGHT INTENSITY ON THE BEHAVIOR OF FISH

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Changes in light intensity markedly affect the behavior of fish, mainly their motor and alimentary activities, their capacity to obtain food, their defense reactions, etc. Since it has a strong effect on the behavior of fish and of the organisms which serve as food for the former, light is one of the important factors determining the nature of the relationship between the fish and its prey, the food availability, switching from one food to another, daily and seasonal vertical migrations of plankton and of fish, etc. All biotic interrelationships in the upper 200 m sea layer are determined by light conditions. The effect of light intensity on the behavior of fish has been only little studied. Methods for studies on the effect of light intensity on the behavior of fish will be described in this chapter.

## Creation of Adequate Light Conditions

In experiments on the effect of light intensity on fish behavior, one should create adequate light conditions. In all experiments conventional electric lamps are used. It should be remembered that light from these lamps shifts somewhat in the direction of long waves as compared with that of natural daylight. Daylight and fluorescent lamps are rarely used. The entire room in which the experiments are carried out may be illuminated, or the experiment may be carried out in lightproof boxes.

Illumination of the entire laboratory where experiments are carried out. Large aquariums or swimming pools for experiments with large organisms should be placed in lightproof premises with walls and floors painted white. One or two deep light projectors are placed in the vicinity. Light from these projectors is directed to the ceiling, from where reflected light falls into the swimming pool or aquarium. I. I. Girska from the Laboratory of Ichthyology in the Institute of Animal Morphology at the Academy of Sciences of the U.S.S.R. studied certain features of the nutrition of sheath-fish in the premises of the Moscow Zoo, by means of this method. She used projectors 35 cm in diameter.

To obtain light of desired intensity, neutral light filters (sheets of clean white paper) were placed on the reflectors. The absorption coefficient of

these filters can be determined with the aid of a selenium photometer. By arranging one or more light filters, the desired light intensity is obtained. The light intensity can be checked with the aid of a photometer (with strong illumination), or can be calculated when the illumination is weak. Changes in light intensity should not occur by switching on electric lamps of varying intensity and at different distances from the swimming pool, as Battl et al. (1936) did, since this method does not ensure uniform light in the various parts of the swimming pool. It is also not recommended to change the light intensity by varying the current intensity, since on diminishing the latter strength, light of the electric lamp is shifted towards red which is often only weakly perceived by fish (especially by marine fish).

**Light conditions in boxes.** It is more convenient to place small fish in special lightproof boxes. This can be done in two modifications. In the first modification (D.S. Pavlov, 1959), used at the University of Moscow, the aquariums were considerably smaller than the boxes—a special recess lined with white tiles and closed by doors, impervious to light. Inside the box the above-mentioned reflector was so placed that it was directed towards the ceiling. A selenium photometer was fixed on the aquarium wall with wires which protruded outside the box. Light intensity was checked by means of paper light filters placed on the reflector.

In the second modifications, developed in the Laboratory of Ichthyology of the Institute of Animal Morphology, special boxes which could be fitted on the aquarium were used. First experiments were carried out with cylindrical boxes made from white tin plates provided with a lid (Figures 1) (Girsa, 1959). Each box had two holes: a side hole with a black sleeve for various manipulations (adding food, taking out nets, etc.), and an upper hole for light. In short-term experiments daylight was sometimes used. More frequently, electric light was used. Light which passed into the case diffused from its gray walls. To change the light intensity, the upper hole was covered with neutral light paper filters. If total darkness was desired, the upper hole was covered with black paper. Light intensity was checked by a photometer placed on the aquarium wall. A circular aquarium was used. A very similar aquarium was employed by Jones (1956).

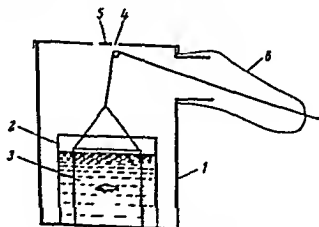


FIGURE 1. Apparatus for studies on the effect of light intensity on fish nutrition (after Girsa, 1959)

1—box, 2—aquarium, 3—net, 4—hole in lid, 5—light filter, 6—sleeve.

Quadrangular aquariums 75-80 cm long, 40-45 cm wide and 40-45 cm high have recently been in use in the Laboratory of Ichthyology of the Institute of Animal Morphology for studies on motor activity and nutrition of predatory fish. The aquariums are covered from above by plywood boxes 90 cm long, 60 cm wide and 55-65 cm high. The bottom of these boxes is removed. They are covered by lids. Side walls of the plywood case have two observation holes. The holes are provided with small black sleeves and sliding lids. The lid of the box also has two holes: one in the center provided on its inner side with a semispherical disperser from opaque glass, and serves for passage of light; the other hole--somewhat on the side--serves for various manipulations. Above the hole for the light a metal reflector with an electric lamp is placed. Light intensity can be varied by means of light paper filters, and checked with a photometer. The outer walls of the case are covered with white paper to disperse the light. If complete darkness is desired, black paper or black cloth are used.

The boxes mentioned above were used in studies on the effect of light intensity on the availability of different edible organisms and their consumption by planktonophages and predatory fish. Studies were also made on the effect of light intensity on motor activity of various fish. Water temperature and its oxygen content should be carefully checked in these experiments.

### Studies on the Effect of Light Intensity on Food Intake by Fish

This method requires that under conditions of desired light intensity, a known number of edible organisms is placed in an aquarium with a known number of fish. After a given time the experiment is discontinued, the number of edible organisms which survive is counted, and the number of edible organisms eaten by the fish is thus determined. The following conditions should be fulfilled:

1. Experimental fish should be hungry but not starved or exhausted. The time required to make the fish hungry varies depending on the fish species and water temperature and must be determined empirically.
2. The experiments should be terminated abruptly, and the fish should be deprived of the capacity to eat at the moment of changing the light intensity. For this purpose a square 6,000 ml net fish pond is used in studies on nutrition of planktonophages. The wire frame of this net is covered with a box. The net is placed in the aquarium, which in its turn is covered with a box. The net contains the fish and the added daphnia or other plankton organisms. By drawing the string attached to the net, the net is lifted out of the water and the experiment is abruptly terminated (during 1-5-2 seconds). If this procedure is omitted, the fish begin to feed voraciously after the end of the experiments, and erroneous results are obtained. In experiments with predatory fish nets are superfluous, since these fish usually do not feed after the end of the experiment. Small surviving fish are counted.
3. Before the experiment the fish and edible organisms should be trained to the light conditions prevailing during the experiment. Usually 30 minutes are sufficient to train fish and edible organisms to light conditions prevailing during the experiment (Girsa, 1959). In recent studies Girsa trained the organisms studied for one hour before the experiments. For experiments with

plankton-eating fish the former are counted and placed in a small vessel filled with water which is placed in the box above the aquarium. Light of the desired intensity is provided, and after a period of adaptation, a string is pulled which throws the vessel with food into the aquarium. This is the beginning of the experiment.

In experiments with predatory fish, small edible fish are placed in a similar vessel or in the aquarium behind a sliding partition. One compartment contains the fish, and another the edible organisms. After adaptation the partition is removed and the experiment begins. The latter is the choice for studies on nutrition of predatory fish, since the behavior of the edible organisms of the beginning of the experiment is undisturbed—a decisive factor (Manteifel' and Radakov, 1960).

4. The ratio between the number of fish and the number of edible organisms they eat is established empirically. The fish should not eat more than their daily ration.

5. The duration of the experiment is determined empirically. The fish should be given time to feed on all or nearly all organisms added. Sometimes the experiments are very short. Five "verkhovka" (*Leucaspis delineta*) in strong light can eat 100 daphnia in 30 sec. In these cases the experiment should be prolonged (Girsa's experiment lasted 5 min). With predatory fish the duration of experiments is usually 15 min. Experiments with little-motile fish (sheathfish) may last 30–60 min.

6. If light conditions permit, behavior of fish and their prey should be watched through observation windows. For observations in weak light the investigator should adapt to the conditions by first sitting in the dark for 30–40 min. According to I. I. Girsa (1959), cancers Harpacticoida are visible at about one lux, still visible at 0.10 of a lux, and invisible at 0.01 of a lux. Saitho fry 6–7 cm long are clear at 0.1 of a lux and discernible at 0.01 of a lux.

Following is a modification of this method. Fish are offered food suspended on very thin silk. The time between the moment of suspension and being eaten is recorded with a stop-watch. The behavior of prey thus suspended clearly varies from that under natural conditions. The results of these experiments are expressed in terms of number of organisms eaten per unit of time (in varying light intensity). To obtain comparative data, fish activity at various light intensities is expressed in percent of activity at optimal light conditions.

#### STUDIES ON THE EFFECT OF LIGHT INTENSITY ON THE MOBILITY OF FISH

For these studies, fish are put in the aquarium, which is covered by a light-proof box, and trained for 30–40 min to the experimental light conditions, their mobility is recorded, by methods which may be mechanical (Jones, 1956; Protasov and Bobyrev, 1959) and photoelectric (Protasov and Mitrokhin, 1959).

**Mechanical recording.** The main part of these devices is a mobile pendulum-shaped contact (Fig. 2). At the end of the pendulum—content a celluloid wing is fixed, and the contact is placed in water. By swimming near the wing the fish causes displacement of water particles, and by shifting the mobile contact from its vertical position brings about closure of the electric circuit. The latter consists of a battery and counter from the automatic telephone exchange, which functions at a current of 20–50 milliamperes. Electromagnetic recorders may be used instead. Closure of the

electric circuit causes a new mark to appear on the counter or is recorded on a moving paper strip of a kymograph. The sensitivity of this recording device depends on the inertia of the pendulum contact and of the moving celluloid wing, as well as on the distance between the mobile and stationary contacts. Reducing the diameter of the ring of the stationary contact and selecting the appropriate size of the celluloid wing according to the size of the fish, narrows the distance between the mobile and stationary contacts thus resulting in an increase in the sensitivity of the recording device. The main drawback of this recording device is the inertia (low response) of the pendulum contact. Many species of small fish (verkhovka, anchovy and others) are unable to move the pendulum from its vertical position so that their movements remain unrecorded.

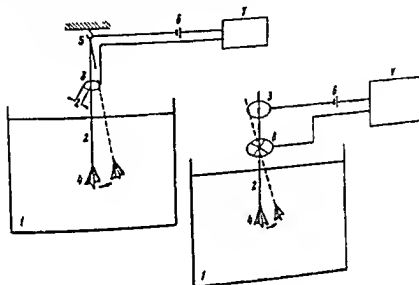


FIGURE 2. Apparatus for mechanical recording of motor activity in fish  
1—aquarium 2—mobile contact, 3—stationary ring-shaped contact, 4—celluloid wing, 5—fine mesh wire 6—battery 7—counter, 8—fastening ring 1—distance between stationary and mobile contacts.

A pendulum contact placed in a position close to equilibrium of its arms is more sensitive (Figure 2 on the right). The stationary ring shaped contact is located around the upper arm of the mobile pendulum contact. The slightest deviations of the celluloid wing leads to closure of the electric circuit. The main drawbacks of this recording device are due to its difficulties in handling, and to its special arrangement of fastening (site of soldering of the pendulum contact to two filamentous wires) which lower its sensitivity somewhat.

Both automatic-telephone exchange counter and kymographs can be used for recording.

Photoelectric recording. These recording devices have no contacts and are therefore very sensitive (Figure 3). They all work on the photoelectric principle.

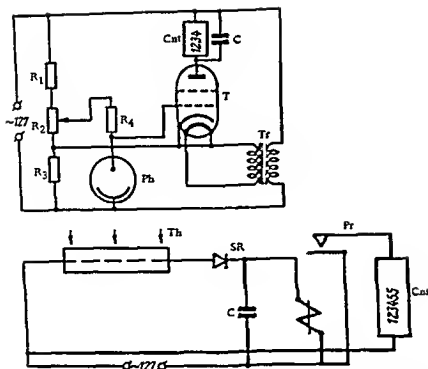


FIGURE 3 Photoelectric device for recording motor activity in fish: top-scheme based on vacuum or gas-filled photocells, bottom-scheme based on semiconductors, Ph - photocell (tsG-1, tsG-2, tsG-3); Cnt - counter (20 to 40 milliamperes); T - tetryon (TGI-0.1/L.3); C - capacity (2-8  $\mu$ F); Tr - transformer for 6.3 volts and 0.5 amperes; R<sub>4</sub> - 1-10 milliohms, R<sub>1</sub> - 3 Kohms, R<sub>2</sub> - 2 Kohms (1 watt), R<sub>3</sub> = 100 ohm, Pr - polarization relay (RP-4), Ts 17,220,287 (actuating current 45-100 ma); Th - thalophide photoresistor, SR - semiconductor rectifier - diode (DGTs-24).

Photoelectric cells or photoresistors (photorelays) are placed along the outer walls of the glass aquarium along a vertical line. At the other side a light source is placed which transmits narrow beams of light to the photorelays. Fish do not perceive the light transmitter because of light filters placed in front of the light source. The filters transmit light within the range from 760 m $\mu$  to 950 m $\mu$ . The photorelay circuit consists of photoelectric cells or photoresistors, of small photomultipliers and telephone counters. When the fish swims through the ray of light, no light falls on the photoelectric cells and photoresistors, and this is recorded by the counter. In Figure 3 simple electric circuits of a photorelay consisting of a photoelectric cell and photoresistor are presented.

#### STUDIES ON FISH BEHAVIOR IN THE DARK AND IN WEAK LIGHT

In some instances it is desirable to study behavior of fish (with organ of vision intact) in the dark or in weak light.

In his studies on spectrum perceived by fish taken from the Barents Sea, Protasov showed that in many fish (cods, haddocks, capelins and others) the spectrum perceived is shorter than in man (in the region of long waves). Exploiting this property of fish we have developed a method for studying the behavior of fish in the dark. By using the part of the

spectrum which is not perceived by fish but is perceived by man, it is possible to watch the behavior of fish in the dark. The experiment is carried out in a dark room. Light from an incandescent electric bulb passes through a Shott or interferential light filter connected with a dispersing lens which transmits broad beams of long-wave light radiations into the aquarium.

In the U.S.A. an attempt has recently been made to use infra-red radiation for studying fish behavior in the dark (Duncan, 1956). This method calls for complicated equipment and also limits the observations to a thin layer of water (because of strong absorption of infra-red rays) which is also strongly warmed by the special lamps used. In this respect the method proposed by us is much simpler and closer to natural conditions.

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## METHODS OF DETERMINATION OF OPTIMAL LIGHT CONDITIONS

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Light, as an ecological and physiological factor, is almost of the same importance to fish as temperature. Numerous vital processes take place under the direct or indirect effect of light (development of reproductive system, vertical and other migrations, intensity and nature of nutrition, and many others). Light is also of great importance for the solution of practical problems in the fishing industry. Let us point out the role of light in various methods of fish catching and as attraction of fish by light, light barriers for some photophobic species (lampreys and eels) as well as for ecologic physiological reactions whose knowledge is indispensable for the fishing industry.

Various aspects of the effect of light on fish can be studied: these range from analysis of photoreceptors to the estimation of the effect of light on the behavior of individual specimens or smaller or larger fish shoals. Attention has recently been directed to the analysis of distribution of fish in light gradients. In these cases the light intensity chosen by the fish can be quantitatively assayed (the fish is offered lights of varying intensity, and mobility of the former is not restricted). The results are expressed in terms of luxes, i.e., area density of light beam of 1 lumen uniformly distributed over an area of 1 m<sup>2</sup>.

For the experimental determination of optimal light intensity, a number of apparatuses producing a light gradient were proposed. The simplest of these apparatuses are chambers in which one end is darkened while the other is illuminated to a certain degree. In such a case we clearly cannot speak of gradients, and these apparatuses allow only for a qualitative estimation of the reaction of fish to light. Such apparatuses were used by Steven (1955), T. I. Privol'nev (1956, 1958) and others.

Of great interest are devices which allow one to obtain stable gradients of light. The first apparatuses for producing a light gradient were apparently two models of Ulliott (1936) in which the light gradient was produced by various means. The first model (Figure 1 on the left) consists of a trough (5) 110 cm long and 35 cm wide. Cardboard shields (4) for reflecting direct rays are fixed at both ends of the trough.

Over the trough a plate made from opaque glass (2) is placed, with blinds (3) from a thin tin-foil arranged at a distance of 3 cm from each other. Light source is an electric lamp (1) on a tripod. To change the intensity



of light the lamp can be moved upwards and downwards light intensity is inversely proportional to the square of the distance from the source of light to the object. Tin foil shields blocked direct rays and formed a light gradient. For determination of light intensity, a selenium photometer graduated in luxes was used. The animals were placed in the trough, which was then exposed to light for a desired period of time. Thereafter, the distribution of animals was recorded and compared with light intensity.

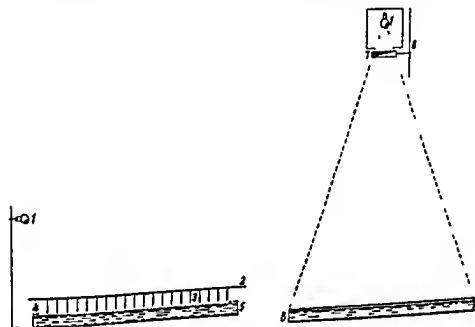


FIGURE 1 Two apparatuses for producing light gradients (after Ulliot, 1936)

1—lamp which can be moved upwards and downwards 2—opaque glass plate 3—vertical tin foil shield, 4—cardboard shield (for protection against direct rays) 5—trough for experimental animals; 6—tripod, 7—wedge-shaped light filter 8—compartment for animals.

In the second model (Figure 1, on the right) a light gradient in compartment (8) was produced by another method. The compartment for the animals is overhung by a light-proof chamber with a lamp (1) inside it. The floor of the chamber has a slit through which light from the lamp passes and falls on the trough with the animals. Immediately below the slit a wedge-shaped light filter is suspended which can be moved upwards and downwards along the tripod to which it is fixed. The wedge-shaped filter is a transparent compartment partitioned longitudinally by a glass plate located diagonally to the side wall. Thus, two prism-wedges are formed. Water in one of these prisms is mixed with India ink or some other stain. Light passing through such filters is adsorbed by the darkened prism, the decrease in light intensity in this case is proportional to the thickness of the layer of colored water. Various segments of the trough will thus obtain light of varying density, and a uniform and stable light gradient will be formed.

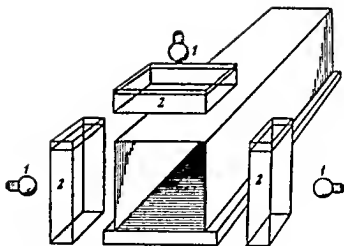


FIGURE 2. Bauers' apparatus (1953)

1—lamps, 2—flat vessels for the protection of one end of the compartment against heating.

Bauers' apparatus for producing light gradients (Bauers, 1953) functions on a different principle. As shown in Figure 2, the compartment for animals (the compartment is 135 cm long and 25 cm wide) is lit at one end by a side light from three lamps. To prevent this side of the compartment from heating, flat glass vessels filled with water are placed between the compartment and the lamps.

To create sharper gradients, Bauers added India ink to the water containing the animals. In this way the extent of light absorption by the water is increased, and it is possible to create a sharp passage from bright illumination to complete darkness in a comparatively short segment. Harris and Wolf (1955) produced sharp vertical light gradient by simply illuminating a jar with water with the aid of an electric bulb suspended above it. If the light intensity on the surface is taken as 100%, it will equal 0.1% at a depth of 32 cm and 0.001% at a depth of 50 cm.

Jones (1955) studied the effect of light on lamprey larvae with the aid of two apparatuses. The first (Figure 3 on top) was used for studies on the behavior of larvae in various parts of the light spectrum of the same light intensity. Larvae were placed in a jar covered with opaque glass. Light from an electric bulb passed through a vessel with water to absorb thermal rays. Various light filters of medium density were placed below this vessel.

For producing light gradients, Jones employed the principle of Ulliot. A gradient was obtained with the aid of a large number of vertical shields placed under an opaque glass plate at a distance of 12 cm from each other (Figure 3 below). The characteristic property of a Jones photogradient is the length of the compartment (4 meters) and the depth of the water (8 cm). This apparatus can be illuminated from both sides by one of the lamps. This forced the animals to move along the chamber in either direction, depending on the strength or location of the light source.

All apparatuses have one thing in common, i.e., the fact that the distribution of animals in the light gradients is recorded visually. It is impossible to count animals in the dark part of the gradient. If the animals are

little mobile, the entire apparatus can be illuminated and the fish counted. In studies on actively mobile fish this method is impractical or very inaccurate. To eliminate these and other secondary errors, we have constructed an apparatus for producing photogradients, and have used it with satisfactory results (Ivlev, 1960)

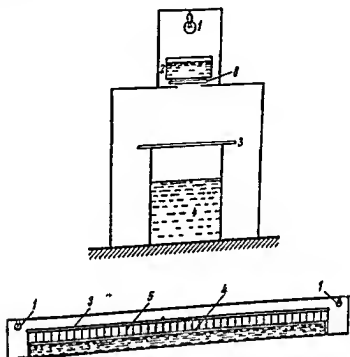


FIGURE 3 Photo-apparatus designed by Jones (1959)

Top—apparatus used for studying the behavior of lamprey larvae in various parts of the light spectrum; bottom—apparatus for producing light gradients. 1—electric lamp; 2—vessel with water for absorption of heat rays; 3—opaque glass, 4—vessel for experimental animals; 5—opaque shields made of black paper.

The main part of our model (Figure 4) is a compartment (1) made of plexiglass, its size 100 cm long, 11 cm wide and 7 cm high. The compartment is placed in a box with heat insulation which prevents passage of light from below and from both sides. Under the floor of the compartment there are 10 K-20 selenium photoelectric cells (2) placed at a distance of 10 cm from each other. With the aid of a ten-way switch (3), light intensity in all 10 cm sections of the compartment can be measured. MP-28 or MS-08 millivoltmeters switched alternately serves as recording device. Two millivoltmeters of different sensitivity are necessary because MP-28 gives accurate results from compartments of low light intensity (to 300 lux) but does not cover the whole range of light intensity studied (from 0 to 800 lux). Hence, when MP-28 was switched on recordings were made over the range from 0 to 250-300 lux with the accuracy to  $\pm 1$  lux, on switching on the MS-08 millivoltmeter recordings were made in the range over 250 lux with an accuracy of  $\pm 5$  lux.

The whole photo-measuring system was calibrated with the aid of a conventional photometer. It was shown, among others, that a 5 cm layer of water in the compartment did not affect the results of measurements. To produce light gradient, the compartment with a ribbon of black plush pasted along its upper edge is covered with a gradient-producing glass which is made in the following way. Photographie plates 13×18 cm are exposed to light and are developed for various periods, so that one plate is completely transparent (immediately placed in a fixer) and the others grow blacker and blacker. Hence, a gradient of blackness is produced. Series of plates with a uniform gradient of light are chosen. These plates are pasted between two glass rods 105 cm long and 13 cm wide.

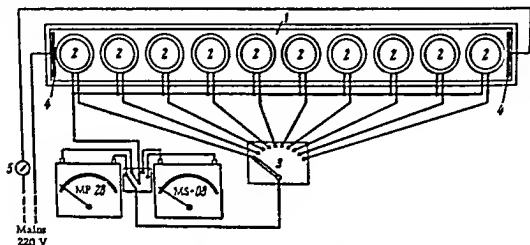


FIGURE 4. Ivlev's apparatus for producing photogradients (1956)

Opaque electric lamp of 200 watts serves as the source of light. The lamp is suspended above the light end of the gradient glass, and may be moved vertically for obtaining light of varying intensity.

In Figure 5 the curve of light intensities obtained in such an apparatus is presented. As can be seen, the curve is close to a straight line and a regular deviation is noted only in the darkened segment.

Finding a method for recording fish distribution in the compartment was of particular importance. This was solved in the following way. At both ends of the compartment two electrodes (4) were inserted (Figure 4). These were made from aluminium plates. The size of the electrodes (11×6 cm) corresponded to the size of the walls of the compartment. With the aid of a switch (5) the electrodes were connected to the circuit of alternate current of 220 volts. Current of this tension gives the animals an instantaneous shock, and they remain immobile. Numerous experiments have shown that during such a shock the fish did not move and their distribution in the compartment was the same as before shock induction. With small organisms such as carp larvae, instantaneous cessation of movements was noted only in water of high mineral content.

Procedure. Before placing the fish in the compartment, the light intensity in each of the ten sections is measured. Ten to 50 fish are then placed in the compartment (depending on their size). One must ascertain that the

distribution of fish in the compartment in the absence of a light gradient is a random one, and that the fish do not concentrate in certain parts of the compartment. The compartment is then covered with the gradient glass and after some time electric shock is produced by switching on contact (5) (Figure 4). The experiments showed that exposure for 2 hours gave a uniform and stable distribution of fish along the light gradient. A 30-40 minute exposure is usually sufficient for a uniform and stable distribution of fish along the light gradient. Several seconds after applying the electric shock the glass is removed and the distribution of fish lying on the bottom of the compartment is determined.

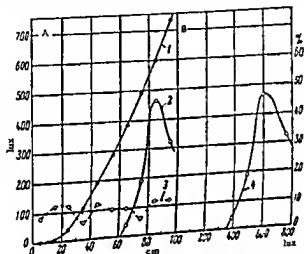


FIGURE 5. Light gradient in the apparatus and fish distribution (A) fish distribution as a function of light intensity (B)

1 - light intensity; 2 - distribution of fish along the gradient; 3 - distribution of fish in diffuse light of 410 lux; 4 - distribution of fish as a function of light intensity. Studies were made on 50 carp fry of an average length of 20 mm. temperature - 18°C (after Ivlev 1960).

Light intensity is recorded during and at the end of the experiment (after all the experimental fish are taken from the compartment). With a stable voltage the results were reproducible. Measurements of light intensity during the experiments sometimes gave low values. This was due to blocking of the light by fish swimming over the photoelectric cells.

The results are interpreted in the following sequence. In Figure 5 curves are presented which show the nature of distribution of light intensity and that of fish (carp) in the compartment. Curves 2 and 3 show the distribution of fish in a light gradient and during ordinary illumination. Curve 4 shows the distribution of fish in relation to light intensity.

To compare results of experiments carried out with different numbers of fish, the distribution of the latter can be expressed conveniently as a percentage of the total number of animals studied.

Significance of the data obtained is determined by repeated experiments. The number of experiments which must be carried out to establish the

significance of these results depends on the reproducibility of the results and the accuracy required.

Fishes which survive electric shock should be discarded.

Gradient of a different type can also be obtained, for example, by illuminating the compartment from both sides. In experiments on the effect of light intensity on fish, their previous history (conditions before the experiment) is of great importance. Our studies showed that fish adapt rapidly to light of varying intensity.

Apparatus of the size mentioned above gave good results with fish 6-7 cm long. It is usually difficult to obtain reproducible results in experiments with larger fish

For other instructions useful in studies on distribution of fish in light gradients, see the chapter written by Ivlev (1962) which deals with methods of studies on thermal gradients.

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# METHODS OF STUDY OF THE REACTION OF SHOAL FISH TO LIGHT

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Studies on the effect of underwater light on the behavior of fish may shed some light on the causes of daily vertical migrations of fish and on causes of attraction of fish to artificial underwater light. Studies on the reaction of fish to light may be carried out in an aquarium the size of which is not less than  $150 \times 600 \times 600$  mm.

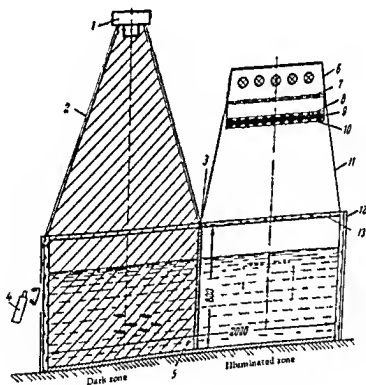


FIGURE 1. Apparatus for studies on the reaction of fish to light

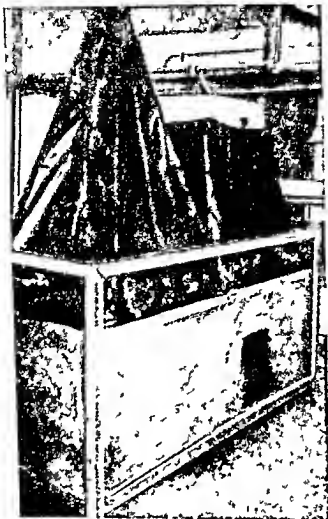


FIGURE 2 Apparatus for studies on the reaction of fish to light

At All-Union Scientific Research Institute for Fishery and Oceanography laboratories, aquariums 200X800X800 mm in size are used (Fig. 1). The aquarium is divided into two halves by a partition made from opaque plastic material. The size of the partition is 800 mm X 400 mm (3). The partition has a slit, (5) 350 mm wide, to allow passage of the fish. The left part of the aquarium is darkened, and the right part illuminated. The illuminating device consists of 9 incandescent lamps (6), opaque glass (7), mask (9) placed between plates (8) and (10) made from frosted glass, screen (13) made from frosted organic glass and an opaque case (11). Frosted glass creates conditions for uniform illumination of screen (13) which, when viewed from beneath, presents a uniformly illuminated surface.

To change the light intensity in the illuminated part of the aquarium, interchangeable masks are used. These constitute unique light filters with numerous openings the area of which is known. Light intensity can also be controlled with the aid of a laboratory autotransformer for feeding current into lamps. The spectral composition of light emitted by lamps changes only



slightly in the process. During lighting regulation it changed from 7800 to 2300\* on decreasing the current from 220 to 120 volts.

Light intensity at the water surface is measured with the aid of a LM 2 luxometer produced by the Moscow cinema electromechanical plant. Light intensity under water is measured with the aid of an underwater VNIRO (All Union Scientific Research Institute of Fishery and Oceanography) photometer consisting of a hermetic source of light with three selenium photoelements switched in parallel, and a sensitive galvanometer.

Camera (2) on a special pyramidal device (1) is placed over the left part of aquarium. Pictures are taken with flash lamp (4) - (exposure 0.0005 sec). With this exposure, the fish have no time to react to the flash and are fixed in their natural position.

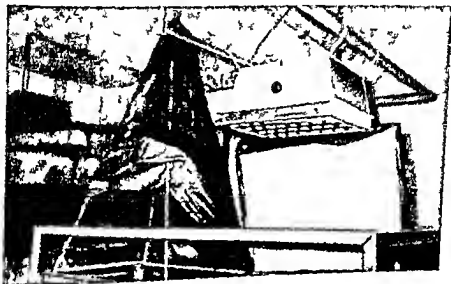


FIGURE 3 Apparatus for studies on the reaction of fish to light (partly disassembled)

To prevent external light from passing into the aquarium the aquarium walls are covered with opaque material (12) leaving only a small window (14) for observations.

A general view of such an apparatus is shown in Fig. 2. The same apparatus partly disassembled is shown in Fig. 3.

In Fig. 3 lamps and a mask are seen. The screen (13) (Fig. 1) of frosted glass is raised. After the experiment is completed the upper parts of both compartments are removed.

Before the experiment 15-20 fish are kept in the aquarium until the appearance of a normal alimentary reaction to food. Larvae of chironomus which can easily be counted are mostly used. Food is given in excess and in various parts of the aquarium to prevent the appearance of reflexes to a certain place of feeding.

During the experiment which lasts 30 min the number of fish present in the illuminated part of the aquarium is counted. The behavior of fish for 10 min before the experiment is not taken into consideration. This period serves for adaptation of the fish to the experimental conditions.

## METHODS OF DETERMINATION OF OPTIMAL TEMPERATURE

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The quantitative estimation of various ecologo-physiological features is of great theoretical and practical interest. The action of environmental factors on a given organism always has a physiological basis, representing a good example of unity of principles and methods of the two main biological disciplines-physiology and ecology.

Temperature is a very important environmental factor. In contrast to many other environmental factors the action of which is usually manifest at their critical concentrations, the biological role of temperature is mainly that of regulation of the rate of physiological processes along the entire biological range. It is clear that this role of temperature is especially pronounced in poikilothermic animals, including fish.

Recently, there has been a renewed interest in certain aspects of the effect of temperature on animals, namely on their distribution under conditions of spatial temperature gradients. The diversity of problems of fundamental importance which can be solved with the aid of this method, as well as the interpretation of the data obtained, have already been described (Ivlev, 1958).

For designating the temperature preferred by animals under conditions of free choice, a multitude of terms have been proposed, preferendum, thermopreferendum, selective or preferred temperature zone, thermotactic optimum, and others. All these terms are used in the Soviet literature, and cause a certain confusion. It would be better if one term were used for designation of the same meaning. Temperature chosen by animals should best be termed selected temperature.

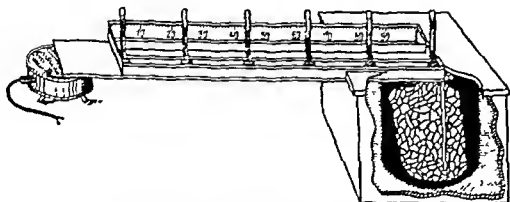


FIGURE 1 Apparatus for producing temperature gradients for studies on aquatic animals. Constructed according to the principle of Henter (from Craevskii and Zabolotskii, 1932)

Studies along these lines resulted in the construction of a number of apparatuses, and led to the solution of other methodological problems. Without dwelling on methods which allow only for qualitative work, we shall describe apparatuses with the aid of which one can obtain satisfactory quantitative results.

The simplest apparatus of temperature gradients for studies on aquatic animals, and fish in particular, was that constructed according to the "classical" principle of Hertter and subsequently widely used in various modifications for the determination of temperature selection by terrestrial animals. The various apparatuses constructed on this principle have been described in detail by N. I. Kalabukhov (1951). The principal part of these apparatuses is an elongated compartment for animals, made wholly or partly from heat-conducting material (metal) heated at one end and cooled at the other end. Such an apparatus adopted for study on aquatic animals is made from a long and shallow metal trough. One end of the trough has a metal sheet immersed in ice, while the other end is heated.

In Fig. 1 an apparatus constructed according to Hertter's principle is shown. The length of the trough was 100 cm, one end of the metal plate was heated on an electric heater, while the other end was cooled in an ice-filled compartment. Temperature in the various parts of the trough was measured with thermometers. Such an apparatus was used by E. Ya. Graevskii and A. A. Zabolotskii (1939) in their studies on temperature selection by aquatic invertebrates, and by N. V. Evropeitseva (1944) in her studies on fish larvae. This apparatus is suitable for studies on little mobile and relatively small animals, and is impractical for studies on large and active fish. This is so because stable temperature gradients in the above apparatus can be produced only with very thin water layers. A temperature gradient cannot be maintained when the water layer is increased to 3-5 cm because of convection mixing and other reasons, such as stirring of the water by the swimming fish.

The first apparatus specially designed for studies on fish and without the drawbacks mentioned above was that of Doudoroff (1938). The Doudoroff apparatus (Fig. 2) consists of a row of 10 communicating octohedral compartments. The total length of all compartments was 203 cm, their width 20 cm, and height 23 cm. The compartments communicate with each other through rectangular slits 5x7.5 cm in size.

**System of water flow.** Three vessels are filled with water (not depicted in Fig. 2). One vessel is filled with relatively hot water, the second with lukewarm water, and the third with cold water. Water is fed into compartments directly from reservoirs or from adjacent compartments. Water is aerated with the aid of a compressor both in the vessels and in the compartments. Water is fed into each compartment in small portions so that there is no flow of water from one compartment into another through the communicating holes. The difference in water temperature in each compartment is 2°C (the total range of temperature gradient is 18°C). The apparatus is placed in a case inside which there are 5 electric bulbs. The lid of the case has a slit for observations on fish behavior. The fish should not be disturbed during the experiment, hence the apparatus is placed in a dark room and the observer remains invisible.

The Doudoroff apparatus seemed apparently faultless. It is nevertheless very complex and difficult to construct and to use. This led to the construction of other models of apparatuses for producing temperature gradients based on different principles.

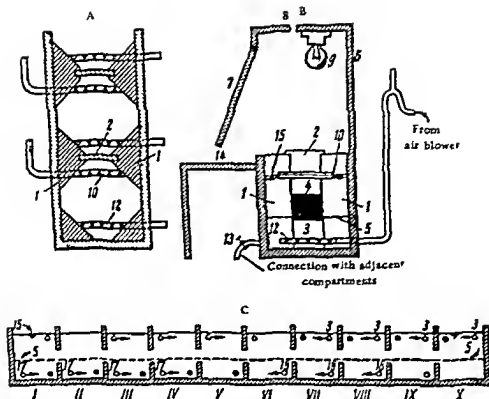


FIGURE 2. Dondoroff apparatus for producing temperature gradients (1956).

A - view from above, B - transverse cross-section, C - longitudinal cross-section showing arrangement of tubes for inflowing (light circles) and outflowing (black circles) tubes, 1, 2, 3 - partitions between compartments, 4 - passages between compartments, 5 - horizontal net, 6 - side wall of case, 7 - flap wall of case, 8 - slit for observations, 9 - lamp, 10 - tube for outflowing water, 11 - reserve tube preventing overflow, 12 - tube for inflowing water, 13 - screw clamp, 14 - lid for outflowing water tubes, 15 - water level, 16 - inlet for cold water, 17 - inlet for warm water, 18 - inlets for lacewarm water, Roman figures (below) indicate octahedral compartments

Comprehensive studies on temperature selection were carried out by Canadian physiologists specializing in fish. Following we shall describe an apparatus for producing temperature gradients as described by Brett (1952).

The main compartment for experimental fish is a rectangular metal tank (97.5 cm long, 97.5 cm high and 50.8 cm wide). The face wall has an iron frame with glass. Cold water is fed through the metal tube, placed at the bottom of the tank. Along the tank walls a coiled copper tube runs from the top down.

Through this tube hot water is fed into tank. Two opposite currents of water (cold water running upwards and hot water running downwards in the coil pipe) created vertical temperature gradient in the tank. Horizontal ropes stretched over the glass plate permit the recording of the distribution of fish along the temperature gradient. Temperature in each water layer is measured with thermometers arranged along the right side wall of the tank.

Brett showed that the vertical distribution of fish in this apparatus is a function of temperature only, and does not depend on pressure, or on geotactic and other factors.

Fig. 3 shows the distribution of fish during shifts in temperature gradient. As shown, the distribution of fish corresponds to the temperature gradient and does not depend on the height of the water layer with the optimal temperature.

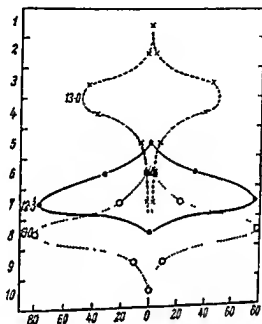


FIGURE 3 Distribution of fish along a vertical temperature gradient. Selected temperature is shifted to layers of varying depth.

Abscissa - number of encounters, ordinates - horizontal water layers in the apparatus

The apparatuses of Kruger (1951) and Schmeing-Engberding (1953) are built on the same principle, temperature gradients are formed by two opposite currents of hot and cold water. These identical models were constructed almost at the same time. Since the authors did not give any references, it is difficult to say which of them was the first to construct the apparatus. This type of apparatus is illustrated in Fig. 4. The glass tube (1) serves as a compartment for experimental fish. The tube is 120 cm long and 4.5 cm in diameter. The tube is closed by rubber plugs with holes for the thermometers. The thermometers lean against the glass along the entire length of the tube (3). Near both ends of the tube there are metallic rets (2) which prevent fish from approaching the tubes for inflowing and outflowing water. The compartment is placed in a metal jacket (4) and is supported on oblique shields (5). The shields serve as a support for the compartment and facilitate the uniform stirring of water flowing through the metal jacket.

Cooled water is fed into the compartment from the left side and flows out through the right tube. Hot water is fed into the metal jacket from the right side and leaves it from the left side through the same tube. Regulating the temperature of water flowing through the compartment and the metal jacket and modifying the rate of flow with the aid of clamps, a desired temperature gradient along the compartment is established. The rate of flow is usually 6 - 8 liters per hour. At such a rate there is no reaction of the fish to water current. Thermometers are usually placed at 10 cm intervals. Temperature difference read on adjacent thermometers is 1 to 3°C. The total range of temperature is within 10 - 15°C.

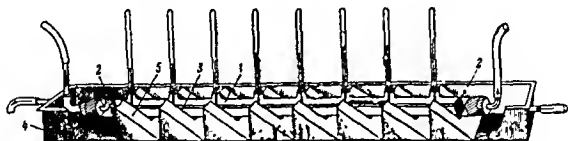


FIGURE 4. Schmeing-Engelberg temperature gradient apparatus (1953)

Comprehensive studies of I.I. Mantel'man (1958) who used the Schmeing-Engelberg apparatus showed that reproducible results could be obtained in experiments with small fish. It is difficult, however, to maintain stable temperature gradients for hours. This requires careful observations and systematic regulation of the preparation.

The last model described is that of Herbert (1956). According to the author, this apparatus produces very stable temperature gradients (an advantage of Herbert's model over the apparatus described above).

Herbert apparatus is depicted in Fig. 5. Compartment (1) for experimental fish has a bend and slopes at an angle of 15 - 30° to the horizontal. Warm water is fed in the right arm (the desired temperature is reached with the aid of an electric heater (3). The cooling device (2) is in the left part of the apparatus. The cooling device consists of a system of tubes with extensions through which cooled water (8) runs. Thermometers (4) are inserted through openings in the upper part of the compartment (as in Schmeing-Engelberg apparatus). The lower part of the compartment is occupied by tube (5) for aeration. The air passes through glass nets (7). Water flows out through tube (6).

Principle of the Herbert apparatus: the sloping position of the compartment causes cold water (owing to its higher density) to flow to the right and downward toward the hot water fed into the right part of the compartment.

Apart from Herbert apparatus, Herbert's device is the simplest of all apparatuses for producing a temperature gradient.

Many of the apparatuses of varying types have a common function. Every experiment begins with the placing of experimental fish in their compartments before creating temperature gradients. Random distribution of fish in the absence of temperature gradient must be ascertained. If several fish are

used in one experiment it should be ascertained that they do not concentrate in certain parts of the compartment. No more than 8-10 specimens should be used in one experiment (this also holds for small fry)

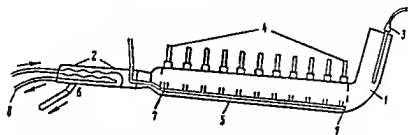


FIGURE 5 Herbert's apparatus (1956)

- 1- glass compartment with bend 2 cooling device 3 electric heater (on the inlet tube)  
4- part of compartment with experimental fish and thermometers, 5 tube for aeration,  
6- tube for outflowing water, 7 glass nets, 8 cold water (inflowing and outflowing)

After some time when the fish are adapted to the new surroundings, and begin to behave normally, the system of heating and cooling is switched on and the desired temperature gradient is created. Recordings are made after a stable temperature gradient has been attained. Since all fish are mobile to some extent, their distribution is expressed in the number of encounters at various temperatures. If the experiments last for 2-3 hours, recordings are made every 5 min. In longer experiments, the intervals are correspondingly prolonged. In Doudoroff's experiments which lasted for 30 min, recordings were made every 6 sec (with the aid of a metronome).

If several fish are used in each experiment, the distribution of fish is best recorded in the form of a graph. The position of the fish at any given moment is marked by pencil on a specially prepared chart.

The data obtained may be processed in various ways. First of all the data should be expressed in the form of a distribution curve. To compare various curves obtained in experiments with different number of fish and registrations, the latter is best expressed in percent of all recordings. The distribution curve is plotted in the following coordinates: temperature is plotted on the abscissa, and the number of encounters is expressed in percent on the ordinate.

The criterion of optimal temperature is modal temperature, i.e. temperature at which the greatest number of encounters were recorded. Biologically, modal temperature is more objective than average temperature. It is clear that when the distribution curve has a symmetric form, the average and modal temperatures coincide. As a rule, however, distribution curves plotted from results of experiments on the behavior of fish in temperature gradients are not symmetrical, and hence modal temperatures should be used.

Modal temperature is usually determined by graphical interpolation, i.e. a point on the line of the abscissa is found which corresponds to the peak of the distribution curve. There are also more accurate methods for determining modal temperature values. These methods are described in numerous hard-books on statistics, and in particular in the comprehensive book of Mills (1938) which has recently been translated into Russian.

Mantel'man (1958) used both modal temperature and "selected temperature zone". Under the latter the author understands a temperature interval with 30% of all recorded encounters. In other words, the selected zone in a normal distribution curve corresponds approximately to the value of the square deviation multiplied by two.

In practice we encounter distribution curves which differ from normal distribution both in range and in asymmetry. These are very important biologically, since the former gives us an idea on euri- or stenometry of the object studied, while the latter, on ecological tendencies towards high or low temperatures. The nature of the data obtained in experiments on temperature selection usually do not lend themselves to standard statistical interpretations of range and asymmetry, since the number of separate variants is insufficient to obtain reliable values. For this reason we have used another method for calculating these criteria. This method is based on the graphic integration of empirical distribution curves (Ivlev, 1958). The following criteria are conveniently used.

Excess  $\beta =$

$$\beta = \frac{1}{M_0} \int_{t_{\min}}^{t_{\max}} n \cdot dt,$$

where  $n$  - number of encounters recorded at the given temperature;  $M_0$  - mode in percent;  $t_{\max}$  - and  $t_{\min}$  - maximum and minimum temperatures delineating the temperature range within which the fish are distributed.

Thermoasymmetry (canting) is equal to:

$$\chi = \frac{\int_{t_m}^{t_{\max}} n \cdot dt - \int_{t_{\min}}^{t_m} n \cdot dt}{t_{\max} - t_{\min}},$$

where  $t_{\max}$ ,  $t_{\min}$  and  $t_m$  maximum, minimum and modal temperatures, respectively.

Integration is done with the aid of ordinary planimeters, since the integral of any curve is equal to the area enclosed between the latter and the coordinates.

As an example we shall describe the curves constructed from the data of Doudoroff (1938), and Sullivan and Fischer (1954). In the first case (Fig. 6) the modal temperature is  $26.4^{\circ}\text{C}$ ;  $\beta = 3.8$  and the asymmetry factor  $X = 1.6$ . In the second case  $t_m = 11.0^{\circ}$ ,  $\beta = 10.7$  and  $X = 0.4$ .

The use of the above criteria allows not only to obtain the value of selected temperature, but also to assess in the form of simple indexes the general nature of the distribution curves.

It is important to know at what temperature the fish had been kept before the experiment. Depending on the aims of the studies, the fish may be taken for experiments directly from water reservoirs where they live or may be kept for some time at the desired temperature for adaptation. For analysis of this problem and references see work of I.I. Mantel'man (1958).



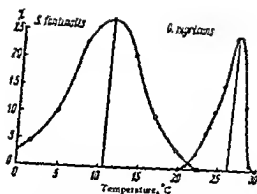


FIGURE 6. Distribution curves of *Salvelinus fontinalis* (after Sullivan and Fischer, 1954) and of *Gleila nigricans* (after Doudoroff, 1938). Vertical lines - modes

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## SIMPLEST METHODS FOR STUDIES ON SOME REFLEXES OF FISH LARVAE AND FRY

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In this chapter certain methods are described which can be used for studies on reflexes of fish larvae and fry under field conditions and on any fishing farm. They deal mainly with the solution of some practical problems of the fishing industry: determination of optimum temperature, flow rate, light conditions and other factors in fish ponds on fishing farms. We shall describe some simple methods of study of the effect of light, water current, temperature and danger from predatory animals on fish larvae and fry. These methods have been elaborated and used by us in experiments with larvae and fry of *Vimba vimba natio carinata* (Pallas) and *Chalcalburnus chalcoides danubicus* (Antipa).

### THE EFFECT OF LIGHT ON LARVAE AND FRY (PHOTOREFLEXES)

The effect of light, both direct and diffuse, on larvae and fish fry, changes with the growth and development of the latter. The effect of light is manifest in the free selection by larvae and fish fry of optimum light conditions. It is clear that experiments on light selection will aid in the investigation of requirements of these organisms for light during their various stages of development. Even with simple observations on the distribution of larvae and fry in illuminated and darkened compartments of an aquarium it is possible to establish clearly pronounced photoreflexes in these organisms (Shkorbatov et al., 1959).

The following is the simplest method for studies on the effect of light on larvae and fry.

A rectangular vessel of 1 000 to 5 000 ml (depending on the age and size of the organisms studied) made from transparent colorless glass is used. The broad lateral walls and the bottom of the vessel are divided into two halves by a mark on the external surface of the glass. One half of the vessel is darkened by covering the outer glass surface with a thin layer of liquid asphalt lacquer (the glass surface should be clean and dry). Paint is applied twice or thrice so that the surface should be light proof. Thus, we obtain an aquarium divided into two parts: illuminated and dark.

The vessel is filled to 4/5 its volume with water. Ten larvae or fry of similar age taken from one water reservoir and from the same tub are placed in the vessel. The vessel is filled with water similar in composition to that in which the organisms were held prior to the experiment. Attention should be paid to the temperature of the water (temperature inside the experimental vessel should be the same as that at which the organisms were kept before the experiments).

Observations are made in various light conditions. In diffuse daylight (at a distance of 1 m from the laboratory window or on the street) and in direct sunlight at various hours of the day. This apparatus is also suitable for experiments with artificial light; in that case the light intensity of the electric bulbs may be changed, as well as the distance from the source of light to the vessel. The vessel should be placed perpendicularly to the light rays to ensure maximum darkening of the dark part and full illumination of the light part of the vessel.

Light intensity of any segment is conveniently measured with the aid of a photometer. In extreme cases an exposure meter used in photography may be employed (for example, exposure meter "Leningrad").

Light intensity in light and dark parts of the vessel is measured before the experiment, and before filling the vessel with water.

Recording of the distribution of larvae and fry in the dark and light parts of aquarium should be commenced 20 - 30 min after placing the vessel in the desired spot. Recordings should be made every 5 - 6 min for one hour. The number of larvae and fish fry in the light and dark parts of the aquarium is counted in the process. Care should be taken that the experimental organisms should not be disturbed by noise, movements of people, etc., factors which may affect their distribution.

The ten recordings made during one hour will give us an idea on the extent of preference by larvae or fry of illuminated or dark parts of the aquarium. The total number obtained from observations in the dark and light parts is taken as 100%. The percent of fish found in the dark or light parts of the aquarium will characterize the effect of light on these organisms. The decimal system of observations (10 organisms and 10 observations) facilitates calculations.

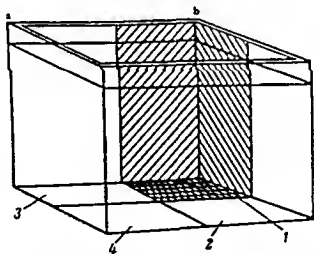


FIGURE 1. Apparatus for studies on photoreflexes in larvae and fry of fish, with the use of four variants of illumination. Glass vessel, the bottom and walls of which are painted for  $1/4$  length with black lacquer (blackened segments are indicated in the Fig. by shaded areas).

A more complex method for studies on photoreflexes involves a procedure by which the experimental organisms have a greater possibility to select light conditions. For this reason, four variants of illumination are produced: dark part, shady part, illuminated part and slightly darkened part.

A rectangular, square bottomed glass vessel of 3,000 - 4,000 ml is used. The bottom is divided into four equal squares and enumerated as shown in Fig. 1. The first square and the corresponding halves of the side walls are covered with a layer of asphalt lacquer. Before the experiment the vessel is filled to  $4/5$  its volume with water in which experimental larvae and fry were kept before the experiment. Twenty organisms are then placed in the vessel. The vessel is then placed in the desired light conditions (diffuse daylight, artificial illumination, etc.). The vessel is so placed in relation to the source of light (the sun, window, lamp) that the rays should fall on the sidewall 'ab' against the first and fourth squares (Fig. 1). In this case squares No. 1 will be darkened, and square No. 2 will be shaded by the shadow falling from the opaque part of wall 'ab'. Square No. 3 will be more illuminated and square No. 4 almost as illuminated as square No. 3. These differences in illumination of the various squares are more pronounced in the sun or on artificial illumination. The background for the noncolored part of the bottom may be changed by placing black, white or colored paper under it.

The distribution of fish larvae and fry in all four zones of the vessel is recorded as described previously.

It is difficult to count simultaneously the 20 larvae and fry present in all four zones of the vessel, since the number of organisms in the various parts of the vessel alters during counting. We thus recommend to take pictures of fry distribution instead of counting them. For this purpose, a 35 mm film camera (for example FED, Zorkii, etc.) should be fixed on a tripod above the vessel. The lens of the camera should permit the taking of pictures from a distance of 50 - 70 cm. The camera should be equipped so that the film can be moved and the shutter cocked and released by a person standing at some distance from the camera. For this purpose the winding lever should be fitted with a wooden drum and a thin strong string wound around it. The string can be wound directly on the winding lever. This enables one to roll the film and to cock the shutter.

To release the shutter, a small wooden board fixed on a pulley with a string on its opposite end may be used (Fig. 2). Winding the string causes the wooden board to lean against the release button and to release the shutter. These devices permit the worker to sit at a distance and not to affect the distribution of fry in the vessel by his presence.

The tripod with camera is properly fixed on the working bench so as not to displace it during the filming. The camera should be focused on all organisms present in the vessel.

Exposure time is not more than 0.01 sec. The first picture is taken 20 - 30 min after beginning of the experiment, and subsequent pictures are taken every 5 - 6 min (altogether not less than 10 pictures should be taken).

Water temperature should be periodically checked in the course of the experiment. When the experiments are carried out in summer and in the sun, and water may become superheated. To avoid this a large volume of water should be used, in addition a large aquarium with cold water (heat filter) should be placed in front of the vessel with the experimental fish.

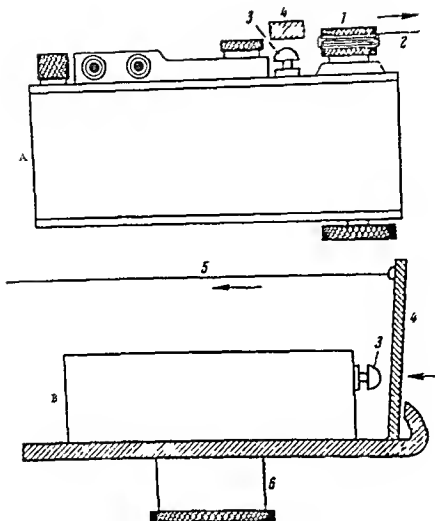


FIGURE 2. Film camera (FED) used in studies on the distribution of fish larvae and fry in zones with different light intensity

A - camera, view from above, B - camera, side view, 1 - winding lever with thread wound around it, 2 - thread of thin string, 3 - shutter release button, 4 - lever for releasing shutter from a distance, 5 - thread for stretching the lever, 6 - lens.

#### THE EFFECT OF RATE OF WATER FLOW ON FISH LARVAE AND FRY (RHEOTAXIS AND LIMNOPHILIA)

The reaction of fish fry to water current is of great interest for ecology and fisheries, as well as for studies on evolution. The appearance of descending instinct in fry of anadromous fish is linked with rheotaxis and limnophilia. It is important that fish breeders should know when to release the fry-of-the-year into the river, since the rapid descent of the fry into the sea, and their survival in the river (maximum protection against predatory fish inhabiting the river in question) depend on correct timing.

The simplest apparatus for studies on rheotaxis in fish is that proposed by A. Ya. Belogurov (1939) and somewhat modified by us for studies on larvae and fry. The apparatus consists of a round basin made from galvanized iron, 40 cm in diameter and 13 cm high (Fig. 3). A metal tube 8-9 mm in diameter is soldered at an acute angle to the side wall about 3 cm above the bottom. This tube protrudes inside the basin for 1-1.5 cm and from outside for 2.5 cm. In the center of the bottom another metal tube 12-15 mm in diameter is soldered in a vertical position so as to protrude above the bottom of the vessel for 9 cm and outside for 3 cm. Along this tube four plates of galvanized iron 13 cm long and 9 cm high are soldered in a vertical position to the bottom of the basin and its walls. Above the plates a tin disk 6 cm in diameter with a hole in its center corresponding to the diameter of the tube (8-9 mm) is soldered.

The basin is painted with a paint indifferent for aquatic organisms (asphalt or white enamel lacquer). The upper circumference of the basin from inside is graduated in cm. The table on which the basin is placed should be provided with a hole for the vertical tube of the basin. The horizontal tube is connected with a tap by means of rubber tubing, and the vertical tube with the sink. Water flow creates a circular current in the basin, with stagnant zones between the plates. The rate of flow and hence the strength of the current is regulated by means of the tap.

The use of this apparatus consists of watching the behavior of larvae and fry at various rates of flow with a stop-watch in hand. The experiments can be carried out on single specimens and on shoals. The above apparatus allows one to solve the following problems:

- 1) how fish fry react to currents, whether they swim with or against the current or whether they seek shelter in stagnant zones at various water flow rates;

- 2) if fry display a rheotactic reflex to swimming against the current the following features should be ascertained
  - a) the speed of movement of the fry against the current at various water flow rates,

- b) maximum rate of flow which can be overcome by fry,
- c) fry fatiguability (how long they are able to resist the current, and the period which they spend in stagnant zones),

- 3) if fry display tendency of remaining in stagnant zones, the minimum rate of water flow at which they leave the stagnant zones should be determined;

- 4) if fry display the descending reflex (tendency to swim with current) one must determine
  - a) at which flow rate this tendency is displayed;
  - b) whether the act of swimming with the current is an active or a passive process, i.e. does the fry actively swim with the current or is it merely carried by it.

To obtain comprehensive answers to these questions repeated experiments must be carried out which reveal the main tendency of fry behavior. Single experiments may yield equivocal and sometimes even contradictory results because of the extreme complexity of reflex movements in fish, which depend on and are influenced by a multitude of external and internal factors.

The speed of current in the apparatus, depending on the rate of flow of the tap water, is determined with the aid of a small floating bar (speed in cm/min) or by measuring the volume of the outflowing water (speed in liters/hour).

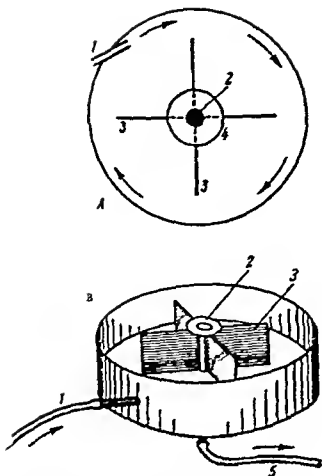


FIGURE 3. Apparatus with circular flow of water for studies on rheotaxis of fish larvae and fry

A - schematic representation from above, B - general view of the apparatus; 1 - tube for inflowing water, 2 - vent of the tube for outflowing water, 3 - vertical plates for creating stagnant zones, 4 - round horizontal plate with tube for outflowing water, 5 - tube with rubber tubing for outflowing water

Volumetric data may be converted into linear data (in cm/min) by appropriate preliminary determinations.

We have constructed an apparatus which imitates the flow of a brook. This apparatus consists of a tub of galvanized iron used in departments of fishing farms for keeping larvae. This tub is divided by three transverse and one longitudinal partition to form one long labyrinth. Length of the tub - 126 cm, its width - 65 cm; height of walls and inner partitions - 15 cm. Due to partitions arranged as shown in Fig. 4, a corridor is formed inside the tub, its width being - 16 cm; This corridor consists of four longitudinal and three transverse bends (inner diameter 2-2.5 cm). The tube is connected with a tap. In the last bend of the labyrinth, 1 cm above the bottom of the tub, three tubes, each 4 cm in diameter, are soldered to the wall of the tub (for outflowing water). Owing to the wide diameter of the water pipe, quite a strong current may be formed in the labyrinth. The rate of outflowing water may be regulated by closing one or two tubes for the outflowing water; 2-3 cm before the tubes for outflowing water a dense metal net or a wooden



frame with gauze are placed (to prevent fry from being carried away with the outflowing water). The water pipe together with the tap is connected by means of a metal coupling to the city water supply line. The height of the tap above the tub may be adjusted from 2 to 50 cm. Owing to this, water falls into the tub from a certain height if this is necessary, to produce sound effects (sound of pouring water).

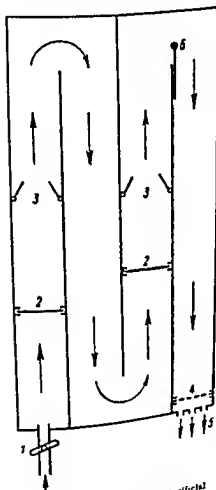


FIGURE 4. Diagram of apparatus (artificial brook) for studies on rheotaxis and limnophilia in fry

1- tap; 2- shields for imitating waterfalls; 3- locks for imitating shoals; 4- protective net preventing fry from being carried away by outflowing water; 5- three water outlets (connected with rubber tubing); 6- flood-gate. Direction of water-flow is indicated by arrows

To create stagnant zones, in this artificial brook, "shoals" and "waterfalls", one lock and one flood-gate are constructed in the first and fifth bends of the labyrinth. Each flood-gate consists of two plates of galvanized tin 8 and 5 cm wide, which are inserted into the grooves of the labyrinth (to imitate small waterfalls when necessary). The "lock" consists of two plates of galvanized tin 23 cm long and 15 cm high. These plates are fixed on hinges one opposite the other. By placing the free ends of the plates at a distance of 2 to 5 cm from each other, a strong current is produced in the middle of the brook, and stagnant zones between the outer surface of the plates and labyrinth walls.

Grooves for the bolts are soldered in the longitudinal partition between the seventh and fifth bends. By pulling them in the direction of the transverse partition (Fig. 4) the current in the fifth bend may be partly or completely obstructed.

The experiments were first carried out without the use of locks and flood-gates. A round wooden float serves for measuring the average speed of water flow in the brook. The float is placed near the first bend which is marked in a certain place on the wall. The time required for the float to pass the entire labyrinth to the protective net is recorded with a stop-watch (the length of this path should be known). This determination is repeated not less than 5 times and the average obtained. The flow rate may be changed by allowing a strong or weak stream to flow from the tap. The speed of outflow from the apparatus may be regulated with the aid of outlets.

To ascertain which reflex prevails in the fry studied (rheotaxis, limnophilia or descending instinct) two types of experiments should be carried out, both with the slowest and maximum rate of water flow.

In the first series of experiments, the fry is placed in the apparatus near the outlets (the slowest speed of water flow is used). If the organism studied swims against the current, the time required to swim the entire length of the labyrinth is recorded. This experiment is repeated several times with different single organisms and with a shoal consisting of 10 - 15 organisms. The rate of water flow is then increased and the same experiments are repeated in the same order.

In this way the following features are determined: 1) the mean speed of movement of the fry against current of various flow rates; 2) maximum speed of water flow which can be overcome by the fry (in cm/min).

If the organisms display strong rheotaxis, they usually never remain the outlet end of the labyrinth (even during the strongest current) but swim to the opposite end of the labyrinth. Fry with limnophilic tendencies or with a descending instinct remain at the outlet and never pass the sixth bend of the labyrinth.

In the second series of experiments the fish are placed near the first bend (near the place where water enters the labyrinth). In such a case the organisms may either remain in this place or immediately swim with the current. The following features are determined: 1) how long the organisms remain near the first bend (at various rates of water flow); 2) the speed with which the organisms swim or are carried by the current (in cm/min). 3) the minimum rate of water flow at which the fry are carried with the current.

Limnophilic fry or those with descending instinct never remain near the first bend, but immediately swim or are carried with the current (even at a low rate of water flow). On the other hand, fry with pronounced limnophilia \* remain immobile for a long time, even at a high rate of water flow.

Experiments of the first and second series are carried out with individual specimens and with groups of organisms, and should be repeated many times at various rates of water flow. Behavior of the fry is determined on the basis of average figures and deviations. Studies of anadromous fish fry in various stages of growth revealed the gradual development of rheotaxis, followed by limnophilia and the appearance of a descending reflex.

Apart from quantitative aspects, these studies also reveal the general behavior of fry under various conditions (varying strength of current) which is of great interest. Locks and flood-gates are used to create conditions resembling those encountered in nature (for example in mountain rivers).

We constructed a small apparatus for studies on the behavior of larvae and very young fry. This apparatus is based on the same principle as the large one which was described earlier. It differs from the latter only in its size (55x30x6 cm) and in some details in the location of partitions (Fig. 5). Experiments with small fry are carried out in the same way as those with larger organisms in the large apparatus.

In experiments with both large and small larvae and fry carried out in the large or small apparatuses, the experimental conditions should be diversified by creating sand, gravel or stony bottoms. This in combination with artificial waterfalls, rapid whirlpools, bolts and flood-gates enables one to obtain an interesting picture of the behavior of fish under a variety of conditions resembling those prevailing in rivers.

\* [Should probably read "limnophilic".]

Fish bred on fishing farms usually live under water temperature conditions differing from those prevailing in rivers and brooks. As a rule, the temperature of water in fish ponds is somewhat higher than that in spawning rivers.

In this connection it is important to know at what temperature these organisms survive and what their optimal temperature is.

The simplest approximate method for determining the upper limit of temperature tolerated by fry is that based on the determination of shock and lethal temperatures. Fish transferred from water with temperatures to which they had been adapted to water with greatly differing temperature display temperature shock: the fish feels ill at ease and assume the 'side position' (lying on side and swimming with the abdomen up). In some instances the fish may recover from such shock; if, however, the difference between the temperatures is too great, the state of shock ends with the death of the organism. Determination of shock and lethal temperatures is of great interest for fish genetics, since it facilitates the selection of organisms resistant to heat and to cold (Samolova, 1941).

Shock and lethal temperatures limits depend on a multitude of factors: a) on the fish species, the physiological state of the fish studied, and in particular on the lability of the central nervous system and its adaptability, and on the regulatory functions of organism to changed environmental conditions, b) on the temperature of water in which the organisms have lived and become adapted before the experiment (Strokanov, 1956).

Experiments on shock and lethal temperatures are regarded as preliminary experiments. Experiments are carried out in two directions simultaneously: 1 - Experimental fry are gradually transferred to various temperatures. Thermostats are used for this purpose. In these experiments water temperature may be maintained at various desired levels: from 15-25°C (the average temperature of brooks from which the organisms are taken) up to 35-40°C. Fry are placed in the thermostats in glass jars filled with well-aerated water. The number of organisms placed in the vessel is determined on their size and species (oxygen requirement) and is so chosen that the organisms should not suffer from oxygen starvation. Water must be

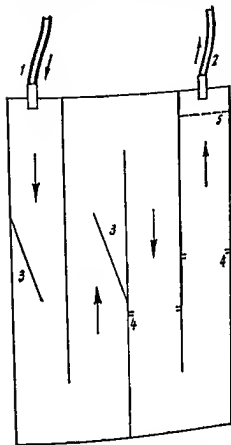


FIGURE 5 Diagram of small apparatus for studies on rheotaxis and limnophilis in fish larvae

1 - rubber tube connected with tap (inflowing water)  
2 - water outlet tube; 3 - stagnant zones formed by detachable plates; 4 - groove for plates imitating waterfalls; 5 - protective net for preventing fish larvae from being carried away with the outflowing water

for temperature selection experiments. Experiments are carried out in two directions simultaneously: 1 - Experimental fry are gradually transferred to various temperatures. Thermostats are used for this purpose. In these experiments water temperature may be maintained at various desired levels: from 15-25°C (the average temperature of brooks from which the organisms are taken) up to 35-40°C. Fry are placed in the thermostats in glass jars filled with well-aerated water. The number of organisms placed in the vessel is determined on their size and species (oxygen requirement) and is so chosen that the organisms should not suffer from oxygen starvation. Water must be

aerated not less than three times a day (by blowing air through a pulverizer bulb) and replaced with fresh water not less than once a day. Water for replacement should be kept in the thermostat so that its temperature should be the same as that in the vessel containing the experimental organisms. Fry are best fed on living daphnia and cyclops.

The initial temperature of water in the experiments is that which is present in brooks for 5 days before the experiments. The temperature in the thermostats is gradually raised, by not more than  $2^{\circ}\text{C}$  a day. The state of the fry is periodically watched. It is convenient to employ thermostats with two doors, one outer metal door and one inner glass door. This enables one to watch the fry without causing any temperature changes in the thermostat. Temperatures at which the following phenomena take place should be recorded: a) isolated cases of shock, b) shock of all organisms, c) isolated death cases, d) death of all organisms studied. If it is necessary to study the effect of low temperatures, the experiments are carried out according to the diagram described, with the exception that in place of a thermostat, a cryostat or refrigerator are used.

11 - The reaction of fry to sudden changes in temperature is also studied. For this purpose, organisms are taken from aquarium with a water temperature to which they were adapted and placed in a series of glass jars with higher temperature. Each jar is filled with water at a temperature higher by  $0.5 - 1.0^{\circ}\text{C}$  than that in the preceding jar. The temperature in the whole series of jars ranges from normal temperature to  $40^{\circ}\text{C}$ .

Small fry (20 - 45 mm long) are conveniently studied in 350 - 500 ml glass jars. To prevent rapid cooling of water the jars should be placed in simple thermostats which consist of a wooden box filled with heat-insulating material (thin sawdust paper, shreds, cotton, oakum etc.) and cork rings for glass jars. The box should be covered with a glass lid. All jars are filled with water at room temperature to which various amounts of hot water are added. In this way a series of jars with a temperature gradient is obtained (each jar is hotter than the preceding one by  $0.5 - 1.0^{\circ}\text{C}$ ). One to two organisms are placed in each jar (this is done with the aid of small gauze net). Their behavior is watched for one hour.

In jars with a high water temperature the organisms usually display shock in a matter of several minutes. In some instances this shock ends in death, in other instances the organisms recover from it. One should record in which vessels (at what temperature) a) the fish die, b) the fish display shock, c) the fish behave normally (no shock occurs). The experiment should be repeated several times, and the temperature in the jars should be measured carefully. If the resistance to cold is also to be studied, similar experiments are carried out, with the exception that the jars contain water with lower temperature than that to which the organisms were adapted. For this purpose, water in the jars is diluted with ice-cold water (at  $0^{\circ}\text{C}$ ).

The results of the experiments on resistance to hot and cold water are supplemented by those on temperature selection. For this purpose we constructed an apparatus depicted in Fig 6 which constitutes a modified version of Schmeing-Engelberding\* apparatus used for studies on the behavior of fish fry in varying temperature gradients (Mantel'man, 1958).

The main part of this apparatus is a metal box (from galvanized iron or zinc sheets) 100 cm long, 10 cm wide and 13 cm high. Metal muffs, 20 cm

\* (See preceding chapter by V S Ivlev )

long, are soldered to both ends of the box. Each muff surrounds the box from four sides from below, from the sides and from the side of the transverse wall. The thickness of the muff is the distance from box wall to the outer muff wall is 3 cm.

Each muff is provided with two tubes 2 cm in diameter the lower for inflowing water, and the upper for outflowing water. The box should be painted with white enamel paint.

The apparatus is placed in a wooden, heat insulated box (felt oakum cotton fine sawdust wood chips etc.). The bottom and walls of the metal box are divided by 4 lacquer marks into 5 segments each 20 cm long. In each segment, at a distance of 1.15 cm from the walls thermometers are fixed. The thermometers should be tested for correct function.

Water supply may vary, depending on technical possibilities heating and cooling devices should be used. If hot and cold running water is available then one muff is connected to the cold water tap, and the other muff to the hot water tap. Under more primitive conditions wooden barrels may be used. Two barrels (capacity of 9-10 buckets) are fixed alongside the apparatus approximately 0.5 m above it. One barrel is filled with hot water and the other with cold water. Each barrel should have a tap fixed at its bottom. The taps are connected with the corresponding lower tubes of one of the muffs by means of a rubber tube. Upper muffs (for outflowing water) are connected with a rubber tube placed in the sink.

The metal box of the apparatus is filled with water at the temperature prevailing in the pond from which the organisms were taken. Hot water flows through the muff and cold water through another. In this way a temperature gradient is produced which is checked by thermometer readings. Each segment should be enumerated.

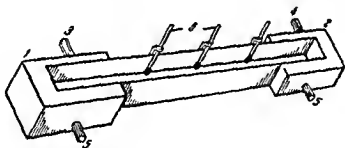


FIGURE 6 Apparatus for studies on temperature selection (fry and larvae thermophila)

1 muff for hot water 2 muff for cold water 3 and 4 outflow tubes, 5 tubes for hot water (left part of the figure) and cold water (right part of the figure); 6 the muffs (three out of 6 thermometers are shown)

Various temperature gradients may be produced by using hot and cold water of varying temperature and by regulating the water flow rates. When the desired temperature gradient has been reached 10 young fish fry are placed in the box. Recordings are commenced after 10-15 min.

aerated not less than three times a day (by blowing air through a pulverizer bulb) and replaced with fresh water not less than once a day. Water for replacement should be kept in the thermostat so that its temperature should be the same as that in the vessel containing the experimental organisms. Fry are best fed on living daphnia and cyclops.

The initial temperature of water in the experiments is that which is present in brooks for 5 days before the experiments. The temperature in the thermostats is gradually raised, by not more than  $2^{\circ}\text{C}$  a day. The state of the fry is periodically watched. It is convenient to employ thermostats with two doors, one outer metal door and one inner glass door. This enables one to watch the fry without causing any temperature changes in the thermostat. Temperatures at which the following phenomena take place should be recorded: a) isolated cases of shock; b) shock of all organisms; c) isolated death cases; d) death of all organisms studied. If it is necessary to study the effect of low temperatures, the experiments are carried out according to the diagram described, with the exception that in place of a thermostat, a cryostat or refrigerator are used.

II - The reaction of fry to sudden changes in temperature is also studied. For this purpose, organisms are taken from aquarium with a water temperature to which they were adapted and placed in a series of glass jars with higher temperature. Each jar is filled with water at a temperature higher by  $0.5 - 1.0^{\circ}\text{C}$  than that in the preceding jar. The temperature in the whole series of jars ranges from normal temperature to  $40^{\circ}\text{C}$ .

Small fry (20 - 45 mm long) are conveniently studied in 350 - 500 ml glass jars. To prevent rapid cooling of water the jars should be placed in simple thermostats which consist of a wooden box filled with heat-insulating material (thin sawdust paper, shreds, cotton, oakum etc.) and cork rings for glass jars. The box should be covered with a glass lid. All jars are filled with water at room temperature to which various amounts of hot water are added. In this way a series of jars with a temperature gradient is obtained (each jar is hotter than the preceding one by  $0.5 - 1.0^{\circ}\text{C}$ ). One to two organisms are placed in each jar (this is done with the aid of small gauze net). Their behavior is watched for one hour.

In jars with a high water temperature the organisms usually display shock in a matter of several minutes. In some instances this shock ends in death, in other instances the organisms recover from it. One should record in which vessels (at what temperature) a) the fish die; b) the fish display shock; c) the fish behave normally (no shock occurs). The experiment should be repeated several times, and the temperature in the jars should be measured carefully. If the resistance to cold is also to be studied, similar experiments are carried out, with the exception that the jars contain water with lower temperature than that to which the organisms were adapted. For this purpose, water in the jars is diluted with ice-cold water (at  $0^{\circ}\text{C}$ ).

The results of the experiments on resistance to hot and cold water are supplemented by those on temperature selection. For this purpose we constructed on apparatus depicted in Fig 6 which constitutes a modified version of Schmeing-Engelberding\* apparatus used for studies on the behavior of fish fry in varying temperature gradients (Mantel'nan, 1958).

The main part of this apparatus is a metal box (from galvanized iron or zinc sheets) 100 cm long, 10 cm wide and 13 cm high. Metal muffs, 20 cm

\* (See preceding chapter by V S Ivlev.)

long, are soldered to both ends of the box. Each muff surrounds the box from four sides, from below, from the sides, and from the side of the transverse wall. The thickness of the muff, i.e. the distance from box wall to the outer muff wall, is 3 cm.

Each muff is provided with two tubes 2 cm in diameter, the lower for inflowing water, and the upper for outflowing water. The box should be painted with white enamel paint.

The apparatus is placed in a wooden, heat insulated box (felt, oakum, cotton, fine sawdust, wood chips, etc.) The bottom and walls of the metal box are divided by 4 lacquer marks into 5 segments, each 20 cm long. In each segment, at a distance of 1 - 1.5 cm from the walls, thermometers are fixed. The thermometers should be tested for correct function.

Water supply may vary, depending on technical possibilities, heating and cooling devices should be used. If hot and cold running water is available, then one muff is connected to the cold water tap, and the other muff to the hot water tap. Under more primitive conditions, wooden barrels may be used. Two barrels (capacity of 9 - 10 buckets) are fixed alongside the apparatus approximately 0.5 m above it. One barrel is filled with hot water and the other with cold water. Each barrel should have a tap fixed at its bottom. The taps are connected with the corresponding lower tubes of one of the muffs by means of a rubber tube. Upper muffs (for outflowing water) are connected with a rubber tube placed in the sink.

The metal box of the apparatus is filled with water at the temperature prevailing in the pond from which the organisms were taken. Hot water flows through the muff and cold water through another. In this way a temperature gradient is produced, which is checked by thermometer readings. Each segment should be enumerated.

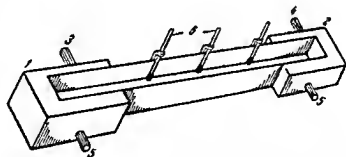


FIGURE 6 Apparatus for studies on temperature selection (fry and larvae thermophila)  
1 - muff for hot water; 2 - muff for cold water; 3 and 4 - outflow tubes; 5 - tubes for hot water (left part of the figure) and cold water (right part of the figure); 6 - thermometers (three out of five thermometers are shown)

Various temperature gradients may be produced by using hot and cold water of varying temperature, and by regulating the water flow rates. When the desired temperature gradient has been reached, 10 young fish fry are placed in the box. Recordings are commenced after 10 - 15 min

a) the distribution of fry in various parts of the box is recorded (their boundaries are marked on the walls by black asphalt lacquer) and b) temperature in these parts is measured. Studies are carried out in the same manner as those on the distribution of fry in experiments on photoreflexes. The number of fry present in the various parts in the given time is counted from the second segment to the last one. Temperature readings are then taken. Such recordings are made every 6 min (ten determinations per hour). Average figures characterize the distribution of fry in each part of the apparatus.

There is no need to measure temperature every 6 min: it is sufficient to take temperature readings at the beginning, in the middle, and at the end of the experiment, and to calculate the average temperature of each part during the experiment.

The results show the temperature preferred by the fry studied (within the temperature range used in the experiment).

Care should be taken that the fry should not be affected by noise, by uneven light or by other disturbances.

More accurate results can be obtained by taking pictures of fry distribution (as described above on p 299 - 300). Procedure: 1) the required temperature and rate of water flow which washes off the terminal muffs are ascertained, 2) the camera is fixed on a tripod and made ready for taking pictures; 3) the fry are placed in the apparatus; 4) 10 min later temperature readings are taken; 5) after another 10 min a photograph of fry distribution is taken. Photographs should be taken by a person sitting in another room or at some distance from the apparatus (remote control of the camera has been described in this chapter); 6) photographs are taken at 6 min intervals on the additional occasions; 7) no pictures are taken for several minutes, and temperature readings are taken; 8) another five pictures are taken; 9) final temperature readings are then taken.

The film is developed and the data are processed as in experiments with visual observations.

The results obtained by the use of the methods for studies on lethal, shock and optimal temperatures on vimba and shemaiia (*Chalcaburnus chalcoides*) fry are presented in the work of Z.P. Sil'chenko (1954), who worked under our guidance.

#### DEFENSE REFLEXES OF LARVAE AND FRY AGAINST PREDATORY ANIMALS

Defense reflexes of fish are of great importance for species preservation, and represent one of the most important factors affecting the return coefficient which characterizes the effectiveness of fish-breeding measures. The bulk of larvae and fry bred in fishing farms and subsequently placed in natural and artificial water reservoirs (ponds, water reservoirs, rivers, lakes etc.) fall prey to predatory organism (dragonfly larvae, beetles, predatory fish).

The percentage of survivors depends on inborn (unconditioned) self-preservation and defense reflexes, and even more on the ability of the larvae and fry to develop conditioned reflexes as a result of the training effect of the environment.

In collaboration with A.I. Ivanova and A.D. Pshedetskaya we developed and tested some simple procedures for studies on the ability of fry to avoid



predatory fish G V Popov (1953) was the first to attempt to combine our method with model experiments (model of predatory fish and induction current stimuli)

For the experiments, 4-5 wooden boxes (app. 60x40x30 cm) with solid walls and metal net flooring are used (2-4 mm mesh) depending on the size of the fry - the latter should not be able to pass through the net

The box is covered with a fishing net or metallic netting (mesh size 5-10 mm) to prevent fry from jumping out of the box during attacks by predatory fish. Instead of special boxes Ses Green or Chalikov incubators may be used

Chalikov apparatuses are better because they have floors and side walls made of metal netting (for better water circulation)

Each box is divided into two compartments by a partition consisting of a wooden frame with a fishing net stretched over it. The partition is fixed in metal or wooden grooves screwed vertically to the inner surface of the side walls of the box. The frame should fit easily in the grooves. The mesh size of the net stretched over this frame is 15-20 mm

The boxes are placed in water (river, breeding or experimental pond depending on experimental conditions) so that they can be periodically watched. Each box is tied with string or soft wire in a floating position (as in the case of Ses-Green apparatuses immersed in water for egg incubation). Each box should contain 10-50 fry, depending on their size. One box serves as control, while other boxes contain one predatory fish

The type of predatory fish used must be chosen in preliminary experiments with various species of predatory fish (ruff, perch, pike and others). The predatory fish should be sufficiently active and attack the fry studied vigorously. The predatory fish should be of small size (so that it may be comfortable in the small box). In the case of small fry, predatory fish 11-15 cm long should be used

All predatory fish should be of similar size. Predatory fish should be kept without food for one day before the experiment

First experiments are carried out in boxes provided with a net partition. The predatory fish is placed in one compartment and cannot penetrate the other compartment because of the net. The fry, on the other hand, can pass freely through the net and swim along the whole box. Fry can escape by swimming into the adjacent compartment which is inaccessible to the predator. Predatory fish are placed in the box for one day together with the fry. The number of fry which survived and those devoured is determined. The experiment is then repeated with the fry which has survived. For this purpose 50 survivors are placed in a box with another predatory fish kept without food for one day

A box with fry and without predatory fish serves as control. The third, final experiment is carried out with fry which has survived both experiments. This time the box is without partition so that the predatory fish can hunt the fry within the entire box. Control experiments with fry which were not trained to escape from predatory fish are carried out. The ability of fry to develop defense reflexes usually becomes evident in the course of three experiments. In some of our experiments with vimba fry two days' training was sufficient for developing defense reflexes. On the third day none of the trained fry was devoured while not fewer than 50% of the untrained fry fell prey to the predatory fish

Each experiment should be repeated not less than 3 - 5 times, to eliminate any deviations associated with individual properties of the predatory fish, of the fry, etc

Below are several variations of the experiments: 1) experiments without the use of nets, 2) experiments in boxes with a stony bottom; 3) experiments with isolated fry.

In the first case the box has no partition even in the first experiment. It is clear that this would lead to great losses, since the fry are devoid of shelter. In such experiments the number of fry used in the experiments should be increased to 60 - 70, depending on the size of the fry and the voraciousness of the predatory fish. In such experiments the fry are trained not to take cover, but to run away from the predatory fish.

In experiments with stony bottoms both reflexes are developed: the ability to run away from the predatory fish, and the ability to take cover in places inaccessible to the latter (on the bottom between stones). Small, flat pebbles (2 - 3.5 cm thick) should be selected. These stones provide an adequate shelter for fry, but do not interfere with the movements of the predatory fish. In these experiments the number of fry which have survived one and two days with predatory fish is counted.

Experiments with single fry are carried out in the following way: Three boxes should be watched simultaneously with a stop-watch in hand. The boxes should not contain stones or partitions. Each box should contain one starving predatory fish. In one box a single untrained fry specimen is placed (fry which has never been in the presence of predatory fish). The second box contains one young fish which has survived one day (in experiment with 50 fry), and the third box contains one young fish which has survived two days in the presence of predatory fish. The time which has elapsed from placing the fry in the box and its being devoured by the predatory fish is recorded. It is difficult to carry out these experiments with slightly active predatory fish and extremely active fry (since it will take a long time for the predatory fish to devour the fry).

In such a case, 3 - 5 fry should be placed in each box.

To obtain a clear picture (by taking average data), experiments with single *untrained and trained fry* should be repeated not less than 10 times.

It is of interest to compare defense reflexes of fry-of-the-year grown under natural and artificial conditions.

This permits to reveal the shortcomings of artificial breeding and to determine the so-called return coefficient with accuracy.

By collecting data on defense reflexes of larvae and fry grown under natural and artificial conditions we shall be able, in the foreseeable future, to solve the problem of eliminating inadequate development of defense reflexes in fry grown in artificial fish ponds.

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**EXPLANATORY LIST OF ABBREVIATED NAMES OF USSR INSTITUTIONS  
AND ORGANIZATIONS APPEARING IN THIS TEXT**

Abbreviation	Full name (transliterated)	Translation
AN SSSR	Akademiya nauk SSSR	Academy of Sciences of the U.S.S.R.
GIZ	Gosudarstvennoe izdatel'stvo	State Publishing House
GOSNIORKH	Gosudarstvennyi nauchno- issledovatel'skii institut rechnogo i ozerного rybno- go khozyaistva	State Institute for Lake and River Fishery Research
IL	Izdatel'stvo inostrannoi literatury	Foreign Literature Publish- ing House
LGU	Leningradskii gosudarst- vennyi universitet	Leningrad State University
MGU	Moskovskii gosudarstvennyi universitet	Moscow State University
ONTI	Ob"edinenie nauchno- tekhnicheskikh izdatel'stv	United Scientific and Technical Publishing Houses
VASKhNIL	Vsesoyuznaya akademiya sel- skokhozyaistvennykh nauk im. V.I. Lenina	All-Union Academy of Agricultural Sciences im. V.I. Lenin
VNIRO	Vsesoyuznyi nauchno-issledo- vatel'skii institut rybnogo khozyaistva i okeanografi	All-Union Scientific Re- search Institute of Marine Fishery and Oceanography